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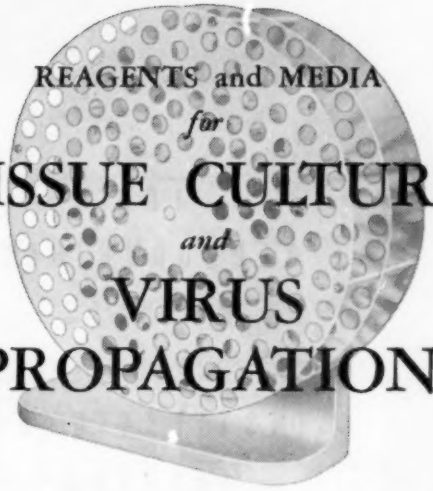

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Experimental Infections of the Bovine Genital System with Trichomonads from the Digestive Tracts of Swine

PAUL R. FITZGERALD, M.S.; A. EARL JOHNSON, M.S.;
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Logan, Utah

INVESTIGATIONS of trichomonads in a variety of animals indicate that these parasites may exhibit a low degree of host specificity. For example, Switzer[6] reported that, when cultures of trichomonads from the nasal and digestive tracts of swine were inoculated into the vaginas of cattle, infections occurred. Similar findings were reported by Fitzgerald *et al.*[3] Hammond and Leidl[5] found that trichomonads cultured from the ceca or feces of swine produced infections when inoculated into the preputial cavities of bulls, and that such infections were transmissible by coitus. The work of Fitzgerald *et al.*[3] indicated that infections by trichomonads of porcine origin in the genital tracts of female cattle may be associated with disturbances of reproduction.

The purpose of this paper is to report these findings in greater detail and to report additional related work concerning experimental infections of the bovine genital tract with trichomonads from the noses, stomachs, and ceca of pigs.

MATERIALS AND METHODS

Eleven grade Holstein-Friesian heifers, 7 grade Jersey heifers, and 1 grade Holstein-Friesian bull were used. The heifers were 13 to 21 months old, and the bull was approximately 2 years old. Five experiments were carried out.

Experiment 1.—Five virgin heifers were inoculated intracervically at estrus with a mixture of semen and trichomonads. The inoculum for 3 of these animals consisted of a mixture of 2 ml. of milk-diluted semen and 2 ml. of a suspension of trichomonads obtained, by culture, from the ceca, noses, or stomachs of pigs. Active, bacteria-free cultures grown in a modified Plastridge medium[2] were used. Before inoculation, the parasites were washed in several changes of sterile, physiological saline solutions. Inoculation was performed by standard techniques of insemination. The other 2 heifers were inoculated similarly, except that the portion of the inoculum containing trichomonads consisted of 1 ml. of a suspension of trichomonads obtained from a culture of *Trichomonas foetus* or a like amount of sediment from a preputial sample obtained from an infected bull. After inoculation, vaginal samples were collected at regular intervals from the heifers by a vaginal pipette. These samples were examined microscopically and by culturing for the presence of trichomonads. Rectal palpations were carried out after an interval of at least 45 days to determine the outcome of the insemination.

Experiment 2.—Six virgin heifers were inoculated intravaginally with material containing trichomonads freshly collected from swine. Of these, 2 heifers were inoculated with 10 to 15 ml. of a dilute suspension of cecal contents in physiological saline solution, and 3 others with 2 to 3 ml. of centrifuged washings from the nasal cavity of an infected pig. The remaining heifer was inoculated with 15 ml. of a saline solution suspension of stomach contents. These materials were obtained from pigs slaughtered in local abattoirs, and heifers were inoculated within three or four hours after collection of the inoculum. Vaginal samples from each of the 6 heifers were collected and examined for trichomonads as described for experiment 1.

Experiment 3.—Five pregnant heifers were each given an intrauterine inoculation with porcine trichomonads or *T. foetus* from culture. Four were inoculated by laparotomy when three to four months pregnant. The other was inoculated through the cervix 34 days after she was bred

From the Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, and the Agricultural Experiment Station, Utah State University, Logan.

TABLE 1—Results of Intracervical or Intravaginal Inoculations of Virgin Heifers with Trichomonas Foetus or with Trichomonads from the Noses, Stomachs, or Ceca of Swine

Group	Heifer (No.)	Breed and age (mo.) at inoculation	Source of trichomonads	No. days between inoculation and last positive sample	Result of insemination
1—Intracervical inoculation at estrus with trichomonad culture and semen	189	J-19	Pig nose	36	No pregnancy
	196	J-13	Pig stomach	77	No pregnancy
	187	HF-17	Pig cecum	35	No pregnancy
	188	HF-16	Bull preputial cavity	20	No pregnancy
	194	J-16	Bull preputial cavity*	108	No pregnancy
2—Intravaginal inoculation with fresh material containing trichomonads	16	HF-21	Pig nose	63	.....
	19	HF-18	Pig nose	46	.....
	184	HF-14	Pig nose	133	.....
	186	J-16	Pig stomach	88	.....
	20	HF-16	Pig cecum	84	.....
	182	J-13	Pig cecum	33†	.....

* Preputial sample used as inoculum instead of culture; † infection resulted from second inoculation; first inoculation resulted in no detectable infection.

naturally. Each inoculum consisted of trichomonads obtained from 5 to 20 ml. of culture medium containing large numbers of actively reproducing organisms. The inoculum was prepared as in experiment 1, and introduced into the lumina of the nonpregnant uterine horns of the 4 pregnant heifers with a needle and syringe. In the animal inoculated by way of the cervix, the inoculum consisted of 10 ml. of a rich suspension of washed trichomonads cultured originally from the nasal tract of an infected pig.

Experiment 4.—To determine the influence of trichomonad infections on responses of heifers to natural or artificial insemination, 8 of those infected with trichomonads in experiments 1, 2, and 5 were inseminated at various intervals after the beginning of infection. Four were inseminated two or three times. The heifers were examined rectally after inseminations to determine whether pregnancy resulted.

Experiment 5.—Transmissibility of porcine cecal trichomonad infections by coitus was tested by mating an experimentally infected heifer with a young bull which had never bred a female. This bull was later bred to a virgin heifer. Preputial samples were collected at regular intervals by the douche method[1] and examined by direct microscopic and culture methods.

RESULTS

Experiment 1.—All 5 heifers inoculated intracervically at estrus became infected (table 1). In the 3 inoculated with porcine nasal, gastric, or cecal trichomonads, the infections lasted 36, 77, and 35 days, respectively, whereas the two infections with *T. foetus* continued for 20 and 108 days, respectively. Return to estrus was delayed in all except the heifer inoculated with swine nasal trichomonads and the 1 inoculated with a culture of *T. foetus.* No pregnancies resulted from inseminations of these animals. There were no marked differences in clinical signs associated with

TABLE 2—Results of Intrauterine Inoculation of Pregnant Heifers with Washed Cultures of Trichomonas Foetus or Trichomonads from the Noses, Stomachs, or Ceca of Swine by Laparotomy *

Heifer (No.)	Breed and age (mo.) at inoculation	Approximate stage of pregnancy (mo.)	Source and estimated No. trichomonads	First and last positive vaginal samples (days after inoculation)	Fate of pregnancy
193	J-18	3	Pig nose 1,500,000	None	Nonpregnant 35 days after inoculation.
200	HF-15	1†	Pig nose ‡	4–56	Nonpregnant 6 days after inoculation.
822	HF-15	3	Pig stomach 10,000	None	Normal pregnancy.
190	HF-17	4	Pig cecum 19,000,000	20–48	Abortion 20 days after inoculation; trichomonads seen in fetal fluids and in fetus.
191	J-21	3	Bovine preputial sample 5,500,000	None	Normal pregnancy.

* In 1 heifer (200) inoculation was done through the cervix instead of by laparotomy; † inoculated 34 days after natural service; rectal palpation at this time indicated that the heifer was pregnant.

the trichomonads of porcine and bovine origin.

Experiment 2.—All 6 heifers inoculated intravaginally with fresh material containing trichomonads from swine became infected although, in 1 heifer, the first inoculation with cecal trichomonads resulted in no detectable infection (table 1). The duration of infection was 63, 46, and 133 days for trichomonads from the pig nose, 88 days for those from the pig stomach, and 84 and 33 days for those from the pig cecum.

Experiment 3.—Of the 4 heifers, three to four months pregnant, given an intrauterine inoculation, 1 aborted 20 days after inoculation, one pregnancy apparently was terminated during the 35 days after inoculation, and 2 had normal pregnancies (table 2). In only 1 of these heifers was an infection demonstrated; the heifer that aborted 20 days after inoculation with swine cecal trichomonads had positive vaginal samples immediately after the abortion and for the succeeding 28 days. Trichomonads were recovered from the fetal fluids and from the stomach, liver, and lungs of the fetus. The heifer returned to estrus two months after abortion. During three subsequent cycles, she was inseminated three times artificially and once naturally, but did not conceive. At necropsy, her reproductive tract appeared normal.

The heifer inoculated by laparotomy with swine nasal trichomonads was found to be nonpregnant when examined 35 days after inoculation, although no abortion had been observed. The 2 heifers inoculated with *T. foetus* or swine gastric trichomonads gave birth to normal, full-term calves. In the heifer inoculated with swine nasal trichomonads by way of the cervix, 34 days after she was bred, a vaginal sample collected four days after inoculation was positive; the infection persisted 56 days. Rectal examination six days after inoculation indicated that the heifer was not pregnant, whereas such an examination just before inoculation had indicated that she was. Whether there had been an error in considering her pregnant or whether she aborted, as a result of the infection or due to tampering with the cervix, has not been determined.

Experiment 4.—Of the 8 heifers, 4 became pregnant as a result of insemination during and after infections with trichomonads (table 3). One of the 4 conceived from natural service 14 days after inoculation with swine nasal trichomonads. Vaginal samples were positive at that time. Rectal examination 49 days after service indicated that she was pregnant, and a

TABLE 3—Influence of Infections of the Genital Tracts of Young Heifers with Swine Nasal, Gastric, or Cecal Trichomonads or with Trichomonas Foetus on Response to Natural or Artificial Insemination

Heifer (No.)	Source of trichomonads and No. days between exposure and last positive vaginal sample	Method of exposure	No. days between exposure and breeding	Kind of insemination	Result of insemination
16	Pig nasal—63	Intravaginal inoculation with nasal washings	14	Natural	Pregnancy
189	Pig nose—36	Intravaginal inoculation with culture	64	Artificial	No pregnancy
			122		
			183		
186	Pig stomach—88	Intravaginal inoculation with stomach contents	27	Natural	No pregnancy
			82	Artificial	No pregnancy
17	Pig cecum—85	Natural service by infected bull	36	Natural	No pregnancy
20	Pig cecum—84	Intravaginal inoculation with cecal contents	32	Natural	No pregnancy
			76	Natural	No pregnancy
			96	Natural	No pregnancy
187	Pig cecum—35	Intravaginal inoculation with culture	29	Natural	Pregnancy
14	*T. foetus*—26	Natural service by infected bull	47	Natural	No pregnancy
			77	Natural	Pregnancy
188	*T. foetus*—20	Intravaginal inoculation with culture	98	Artificial	Pregnancy

vaginal sample collected at the same time, 63 days after inoculation, was positive for trichomonads. However, a sample collected 123 days after inoculation was negative. The outcome of the pregnancy is unknown. Another of the 4 became pregnant when bred 29 days after inoculation with swine cecal trichomonads, while vaginal samples were still positive. In the other 2 heifers, pregnancies resulted from services 77 and 98 days after exposure to infection with *T. foetus*; approximately 7 and 11 weeks, respectively, after vaginal samples had ceased to be positive.

Pregnancy failed to develop in 3 heifers infected with swine cecal or gastric trichomonads and inseminated during the infection or shortly thereafter, and in 1 heifer infected with swine nasal trichomonads, although she was inseminated three times one to five months after the vaginal samples became negative.

Experiment 5.—In the experiments involving transmission of swine trichomonads by coitus, the young bull remained negative after his first exposure with a heifer experimentally infected with swine trichomonads; however, the mating occurred while she was not in estrus. Infection did occur from a second mating with the same heifer, 14 days later, while she was in estrus. The first preputial sample, taken 15 days later, contained trichomonads, and the bull remained positive for four months, after which samples were consistently negative. While infected, the bull bred a virgin heifer. The first vaginal sample, collected 16 days after exposure, was positive; samples from this heifer were positive when last tested, 85 days after breeding.

DISCUSSION

These results confirm the findings of Switzer [6] that porcine trichomonads are capable of becoming established in the vaginas of cattle. Switzer found that inoculation of a culture of swine nasal trichomonads into the vagina of a heifer resulted in an infection lasting three weeks, whereas a similar inoculation with a culture of cecal trichomonads resulted in an infection lasting 92 days. A mild catarrhal vaginitis was observed in both animals five and six days after the inoculations. No attempt was made to determine the effect of these infections on fertility.

In the present study, infections with nasal trichomonads lasted somewhat longer than those reported by Switzer, whereas two of the three infections with cecal trichomonads were shorter in duration, and one was similar to that reported by Switzer. The fact that pregnancies did not occur in any of the 3 heifers simultaneously inseminated and inoculated with porcine trichomonads indicates that the infections interfered with the early stages of development of the embryo.

The trichomonad infections resulting from inoculation with freshly collected cecal or stomach contents or nasal washings from pigs, in which trichomonads were much less numerous than in cultures, demonstrate that high concentrations of these parasites from pigs are not necessary to initiate infections in the bovine vagina. The results of these inoculations also indicate that natural infections of the bovine genital tract with porcine trichomonads may occur when pigs and cows are kept in the same or adjacent areas.

The abortion which occurred 20 days after intrauterine inoculation of swine cecal trichomonads into a heifer four months pregnant (experiment 3) apparently was caused by the infection, as indicated by the presence of trichomonads in the fetal fluids and in the fetus. The relationship of the other interruption of pregnancy to the inoculation of trichomonads is questionable, because of the lack of any evidence that infection occurred. The occurrence of normal pregnancies in 2 heifers given intrauterine inoculations with trichomonads (table 2) might have been associated with the timing of the inoculation. The time of invasion of the uterus by *T. foetus* in natural infections is unknown, but it probably occurs within the first few weeks of pregnancy. Conditions for multiplication of trichomonads in the uterus might be less favorable at three months of pregnancy than earlier.

Although the number of cases was too small to permit definite conclusions to be drawn, the results show that infection of the female genital tract with trichomonads from swine frequently was accompanied by infertility during and for some time after the period when these parasites could be found in the vagina.

Transmission of an infection with the swine cecal trichomonad by coitus, from a heifer to a bull and from the bull to a heifer, confirms the report of a similar

finding by Hammond and Leidl.[4,5] They reported infections in 2 heifers, initiated by natural service of an experimentally infected bull, with durations of 62 and 67 days, as compared with a duration of 85 days in the single similar case in this study (exper. 5). Spontaneous recoveries of infected bulls apparently occurred in both studies—four months after the infections began, in this study; in the former study, recoveries occurred in 2 bulls after 11 and 30 days, with a reinfection of 1 bull lasting 45 days. These authors reported that a second infection in the second bull and original infections in 2 others were present 75 to 200 days after exposure; these infections apparently were chronic.

With respect to transmissibility, course of infection, and pathogenicity in cattle, the swine cecal trichomonad appears to resemble *T. foetus*. This also may apply to trichomonads from the noses and stomachs of pigs, although the evidence is not as complete for these as for the cecal form.

SUMMARY

One bull and 18 heifers were experimentally infected with trichomonads from the noses, stomachs, or ceca of pigs or with *Trichomonas foetus*.

In 3 virgin heifers inoculated intracervically at estrus with a mixture of milk-diluted semen and trichomonad cultures from swine, infections lasting 35 to 77 days resulted. Two heifers similarly inoculated, except that *T. foetus* from cultures from a preputial sample was used instead of the porcine trichomonads, had infections lasting 20 and 108 days. None of the heifers became pregnant.

In 6 heifers inoculated intravaginally with trichomonads collected directly from pigs, infections with nasal trichomonads lasted 63, 46, and 133 days, the infection with stomach trichomonads lasted 88 days, and the two with cecal trichomonads lasted 84 and 33 days.

Intrauterine inoculation of porcine cecal trichomonad culture by laparotomy into a heifer, four months pregnant, was followed by an abortion 20 days later; trichomonads were found in the fetal fluids and fetus. After a similar inoculation of porcine nasal trichomonads into a heifer, three months pregnant, no positive vaginal samples were observed, but the heifer was not pregnant 35 days later. Pregnancies of three months' duration were not interrupted in 2 heifers inoculated similarly with porcine gastric trichomonads or *T. foetus*, and there was no evidence of infection.

A bull became infected with swine cecal trichomonads as a result of breeding an experimentally infected heifer; this bull then transmitted the infection to a virgin heifer by coitus. The bull remained positive for four months.

Of 8 heifers naturally or artificially inseminated during or after infections with trichomonads of swine origin or with *T. foetus*, 2 conceived when bred during an infection with nasal or cecal trichomonads, 2 conceived several weeks after the last positive vaginal samples after infections with *T. foetus*, and the remaining 4 did not conceive.

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Leptospira Canicola Infection in a Newborn Calf

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SINCE *Leptospira canicola* was first isolated from a sick dog in 1931 by Klarenbeek and Schüffner,[15] its widespread distribution in canine hosts has been demonstrated in various parts of the world.[11] That infected dogs may serve as potential sources of *L. canicola* infections in human beings has been repeatedly demonstrated.[21] Recently, the broader significance of canine species in the infectious cycle of leptospirosis in the larger domestic animals has been recognized.[4, 8, 17, 25, 30]

In Israel, *L. canicola* was isolated from cattle and swine during various outbreaks of leptospirosis in these hosts.[20, 26] Either jackals or dogs served as the primary reservoir of infection, although subsequent infections of human beings with *L. canicola* were traced to swine carriers. Serological evidence of infections with this same serotype was also obtained in horses, mules, and donkeys located in the epizootic areas.

More recently, Kmety [16] isolated *L. canicola* (among other serotypes) from presumably normal swine at a slaughterhouse and demonstrated the potential *L. canicola* infection hazard in abattoir employees. Similarly, Coghlan *et al.*[5] reported cases of *canicola* fever in piggery workers which were attributed to swine as the source of infection.

In this country, the infectivity of *L. canicola* for large domestic animals was demonstrated in studies of an epidemic in Georgia of *canicola* fever in man.[30] The epidemic was associated with a history of bathing in a small creek around which cattle and swine were pastured and which also was used by local dogs. During the course of this investigation, leptospiras, subsequently identified as *L. canicola*, were isolated from human beings, dogs, and a sow. In addition, serological evidence of *L. canicola* infections in cattle was obtained.

Other studies in the United States have provided serological evidence of *L. canicola* (as well as other serotypes) in cattle.[6, 12, 29, 30] However, the etiological demonstration of leptospirosis in cattle has been exclusively restricted to *Leptospira pomona*.

A bacteriologically verified *L. canicola* infection in a calf is reported in this paper.

Case Report

A grade Hereford cow and her day-old calf were presented to the Large Animal Clinic, School of Veterinary Medicine, Alabama Polytechnic Institute, Auburn, for treatment of a teat wound. The owner had noticed that the calf had "bloody urine." On examination, the calf showed jaundice, listlessness, and hemoglobinuria. On the following day, it was more depressed and had difficulty in standing. Since leptospirosis was suspected, urine was collected for bacteriological studies. Blood samples for serological studies were obtained from the calf and the dam at once, and again 11 weeks later.

The serum bilirubin determination for the calf revealed 66 mg./100 ml. of combined protein bilirubin with no free bilirubin. To eliminate the possibility of this combined bilirubin being a result of hemolysis of erythrocytes because of the presence of isoagglutinins transferred from the cow, blood of the dam and calf was cross matched. Incompatibility was not demonstrated. Destruction of erythrocytes and the resulting icterus were demonstrated by the following hematological findings: erythrocytes, 1,940,000 per cubic centimeter of blood; leukocytes, 17,550 per cubic centimeter of blood; differential leukocyte count—63 neutrophils, 34 per cent lymphocytes, and 3 per cent monocytes; packed cell volume, 15 per cent; icterus index, 153.5 units; and hemoglobin, 3.2 Gm./100 cc. of blood.

Until the calf was 3 days old, the only treatment was the force feeding of the dam's milk. On the third to fifth days, it was given 500 ml. of whole blood and 350 ml. of 5 per cent dextrose intravenously, daily. Hemoglobinuria was noticed until the sixth day, when the maximum body temperature was 103.6 F., and increased respiration and pulse rates were observed. Penicillin (4,000 units) and dihydrostrep-

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tomycin sulfate (1 Gm.) were injected subcutaneously on the sixth, seventh, and eighth days. The calf became afebrile on the seventh day, and the cow and calf were returned to the herd on the fifteenth day.

During the past year, one abortion was known to have occurred in the herd of 25 grade dairy and beef cattle, but other abortions could have occurred without being noticed. The herd owner stated that, just prior to the incident described, a dog on the farm had died from a "liver disease." Unfortunately, the herd was dispersed before samples of blood for serological tests could be obtained. However, 3 of the cattle were located and tested.

MATERIALS AND METHODS

Isolation Procedures.—Mature chinchillas, weighing approximately 400 Gm., have been used routinely for the isolation of leptospiras at the Agricultural Experiment Station of the Alabama Polytechnic Institute. They are highly susceptible and have been used with considerable success for the isolation of *L. pomona* from body fluids.[20]

The chinchillas were usually inoculated with 0.5 ml. of urine. Their temperatures were recorded daily, and heart blood for culture was obtained on postinoculation days 3 and 5, and again when the animals showed febrile responses. One drop of each blood sample was inoculated into each of two tubes of Chang's semisolid medium[7] containing 10 per cent rabbit serum. Cultures were incubated at 25 C. and examined for the presence of leptospiras by dark-ground microscopy every two days during the first week, and at irregular intervals thereafter.

Serological Tests.—Serums were examined for the presence of leptospiral agglutinins by the microscopic agglutination ("agglutination-lysis") test, using living Leptospira organisms.

Identification of Organism.—The organism isolated was identified at the Walter Reed Army Institute of Research. Antigenic characteristics were determined on the basis of results of cross-agglutination and agglutinin-absorption procedures. The techniques employed in the preparation of rabbit antiserum and in the conduct of cross-agglutination and agglutinin-absorption tests are described in detail elsewhere.[7]

RESULTS

Identification.—The 2 chinchillas inoculated with urine from the sick calf died on postinoculation day 7 with signs and lesions similar to, but more pronounced than, those observed with *L. pomona* infections in these hosts.[20] A severe hemorrhagic enteritis, generalized icterus, and hemorrhagic foci on the lungs were some of the more outstanding gross lesions. Leptospiras were isolated from chinchilla blood samples obtained on postinoculation day 5.

TABLE 1—Comparison of Microscopic Cross-Agglutination Reactions of Serotype Antiserums Against Leptospira Type Strains

Rabbit antiserums*	Reciprocal of titer against antigen								
	Wiggins	*Canicola*	*Jonsis*	*Patane*	*Malaya*	*Sumneri*	*Benjamin*	*Schüffneri*	*Ballum*
L. pomona	—	—	—	—	100	—	—	—	—
L. grippotyphosa	—	—	—	—	—	—	—	100	—
L. javanica	—	—	100	400	—	100	—	—	100
L. canicola	102,400	25,600	6,400	6,400	6,400	25,600	6,400	6,400	1,600
L. jonsis	25,600	25,600	102,400	25,600	6,400	25,600	6,400	6,400	100
L. patane	25,600	25,600	25,600	25,600	6,400	25,600	25,600	25,600	1,600
L. malaya	6,400	6,400	1,600	1,600	25,600	6,400	6,400	6,400	—
L. sumneri	25,600	6,400	6,400	1,600	6,400	102,400	1,600	1,600	1,600
L. benjamin	6,400	6,400	6,400	6,400	25,600	6,400	25,600	6,400	100
L. schüffneri	1,600	6,400	1,600	1,600	6,400	6,400	25,600	25,600	—
L. ballum	1,600	400	—	1,600	—	1,600	—	—	102,400
L. icterohaemorrhagiae AB	6,400	6,400	400	400	6,400	25,600	400	400	6,400
L. icterohaemorrhagiae A	6,400	6,400	6,400	6,400	6,400	6,400	6,400	1,600	—
L. mankarso	6,400	6,400	—	—	6,400	1,600	—	100	100
L. smithii	400	100	—	—	1,600	400	—	—	—
L. birkini	6,400	6,400	1,600	1,600	1,600	6,400	1,600	6,400	—
L. naam	1,600	6,400	100	—	1,600	1,600	1,600	1,600	—
L. pyrogenes	1,600	400	—	100	—	100	—	—	100
L. zanoni	1,600	400	400	—	6,400	102,400	—	—	—
L. alexi	1,600	400	—	400	—	100	—	—	400
L. robinson	—	—	1,600	6,400	—	—	—	—	—
L. abramis	400	100	1,600	100	—	1,600	100	100	—
L. biggis	100	—	100	—	100	400	—	—	400
L. hamptoni	100	100	100	100	—	100	—	—	100

* No agglutination at 1:100 (= —) against *L. andaman*, *L. semaranga*, *L. djatzi*, *L. hyos*, *L. djasiman*, *L. sentot*, *L. autumnalis* AB, *L. medanensis*, and *L. celledoni*. Homologous titers were generally 1:25,600, occasionally 1:6,400 and 1:102,400.

TABLE 2—Results of Cross Agglutinin-Absorption Tests on Calf isolate Wiggins and Leptospira Canicola (Hond Utrecht)

Rabbit antiserum		Reciprocal of titer against antigen	
Against	Absorbed with	*L. canicola*	Wiggins
L. canicola	Unabsorbed	25,600	25,600
	L. canicola	— *	—
	Wiggins	400	—
Wiggins	Unabsorbed	25,600	25,600
	L. canicola	—	—
	Wiggins	—	—

* — = neg. at 1:100 dilution.

The isolated culture (strain Wiggins), used as antigen, was initially screened against 18 serotype rabbit antiserums routinely used at the Walter Reed Army Institute of Research.[13] These tests demonstrated the serological affinities of strain Wiggins and members of the *canicola* serogroup. To elucidate other possible antigenic relationships, the comparative cross reactivity of strain Wiggins, the type strain of *L. canicola* (strain Hond Utrecht), and other type strains[3] antigenically related to *L. canicola* were studied. For this purpose, 16 different serotype antiserums, in addition to the 18 screening antiserums, were used. The serological cross-reactive patterns of strains Wiggins and Hond Utrecht were essentially indistinguishable, but differed qualitatively and quantitatively from the reactions observed with type strains of *Leptospira jonsis*,[3] *Leptospira malaya*,[3] *Leptospira sumneri*,[3] *Leptospira benjamin*, *Leptospira schüffneri*, *Leptospira patane*,[24,29] and *Leptospira ballum* (table 1).

Although strains show similarities in their cross-agglutination reactions, they may not be antigenically homologous. Observations in this regard were made in previous serological studies,[1,2] and are also evident in the data presented (table 1). For example, the serological reactions of *L. schüffneri* and *L. benjamin* are remarkably parallel, although they are distinct heterologous serotypes. Therefore, for the definitive typing of the Wiggins strain, reciprocal agglutinin-absorption studies were conducted with the calf isolate and *L. canicola*, strain Hond Utrecht. Results of "mirror tests" (table 2) show minor antigenic differences between strain Wiggins and the type strain of *L. canicola*. The former strain contains part, but not all, of the antigenic components of strain Hond Utrecht. According to current classification criteria, these differences are not sufficiently disparate to warrant taxonomic recognition of the Wiggins strain.[23] Consequently, strain Wiggins is classified as a homologous serotype of *L. canicola*.

Serological Studies.—The initial serum samples obtained from the 2-day-old calf and its dam did not show agglutinins against *L. pomona* and *Leptospira autumnalis* and, since infection with *L. canicola* was not considered, this antigen was not used in the first microscopic agglutination tests. When the isolate was identified as *L. canicola*, second specimens were obtained, 11 weeks after the first, from the dam and calf and from a dog at the same farm. The serums were tested against 18 antigens, including strain Wiggins.[13]

No agglutinins were detected in the dog serum. The calf serum showed a titer of 1:100 against strain Wiggins and a partial reaction at this same dilution against *L. canicola*. The serum of the dam showed titers of 1:1,600 against strain Wiggins, 1:400 against *L. canicola*, and 1:400 against *Leptospira pyrogenes*—reactions that are fairly typical in *L. canicola* infections. Serum from 1 cow that had been a member of the herd had a specific titer of 1:1,000 against *L. canicola*. The serological observations, in addition to the culture typing results, provide unequivocal evidence that the calf was infected with *L. canicola*.

DISCUSSION

Although Podgwaite *et al.*[19] isolated *L. pomona* from aborted bovine fetuses, other workers[9,10,18] have not been able to demonstrate leptospiral invasion of fetal tissues in experimental infections of pregnant heifers or cows with *L. pomona* or other serotypes. The etiological demonstration of *L. canicola* infection in a sick newborn calf, coupled with the demonstration of significant homologous agglutinins in the dam, would strongly indicate that the infection occurred *in utero*, and that leptospiras can penetrate the bovine placenta.

Of greater public health and livestock sanitary significance is the demonstration of *L. canicola* infection in cattle. Unfortunately, it was not possible to elucidate the epidemiological factors in this case. The history of an abortion in a cow, and the contacts with a dog with a "liver disease" that died shortly before the calf was

born, may be significant. The demonstration of a specific titer against *L. canicola* in another member of the herd affords evidence that the reported infection was not a singular episode. In view of the close association of livestock with dogs, a species that shows a high prevalance ratio of *L. canicola* infection,[4] it is reasonable to expect the presence of this serotype in the various species of livestock. Also, dogs can be the recipients of infection with serotypes that are usually associated with livestock.[17]

Preliminary observations on experimentally induced infections in 2 calves, using the isolated agent, indicate that *L. canicola* can readily establish infection in these hosts. This confirms Van der Hoeden's [27] observation of the localization of *L. canicola* in the kidneys of infected animals. The extent and duration of leptospiruria in cattle infected with *L. canicola*, an obviously important factor in the epidemiology and epizootiology of this disease, require additional study.

Cattle, as well as other domestic animals, can be infected with a wide variety of leptospiral serotypes.[28, 31] In many areas of the world, infections in cattle are primarily produced by serotypes other than *L. pomona*.[5, 28, 31]

In the United States, agglutinins against *Leptospira grippotyphosa* and *Leptospira sejroe*, in addition to *L. pomona* and *L. canicola*, have been demonstrated in cattle in a limited number of surveys.[12, 29, 30] However, in diagnostic, epidemiological, and control measures, attention has been primarily directed to *L. pomona*. As previously reported,[17] "the testing of livestock serum solely against *L. pomona* antigen may not detect the presence of agglutinins provoked by antigenically dissimilar serotypes." The use of additional antigens may uncover a significant percentage of reactors that have previously been missed. In an analogous survey in dogs, the use of multiple antigens uncovered twice as many reactors as would have been disclosed if test antigens were restricted to *L. canicola* and *Leptospira icterohaemorrhagiae*.[17]

Recent studies [22] have disclosed wildlife reservoirs of *L. grippotyphosa, Leptospira mitis* (synonym, *Leptospira hyos*), a member of the *hebdomadis* group, and *L. autumnalis*—serotypes that have been commonly associated with bovine leptospirosis in other countries.

At the Walter Reed Army Institute of Research, the following antigens have been routinely used in serological tests for leptospirosis in livestock: *L. pomona, L. canicola, L. icterohaemorrhagiae, L. grippotyphosa, L. sejroe*, and *L. autumnalis*. Recently, in view of these findings, *L. mitis* has been included in the screening antigens. Samples are also frequently checked against other leptospiral antigens used in the serological testing of serums from human beings.[13]

Vaccines, using *L. pomona* bacterins, are now being extensively used in this country for the control of leptospirosis in livestock. However, these vaccines may have no prophylactic value for leptospiral infections incited by *L. canicola* or other heterologous serotypes. A further study of this problem, as well as the other public health and livestock sanitary aspects of leptospirosis in domestic animals, is indicated.

Summary and Conclusions

A Leptospira isolated from the urine of a sick calf, 2 days old, was identified as a homologous serotype of *Leptospira canicola*, on the basis of cross-agglutination and agglutinin-absorption procedures. Specific antibodies were subsequently demonstrated in the calf and its dam.

The infection in the calf was probably contracted *in utero*.

One abortion had occurred in the herd of origin and serological tests indicated that other members of the herd may have been infected with *L. canicola*.

This, as well as other serotypes recently demonstrated in wildlife hosts, may be an important etiological agent of leptospirosis in American livestock.

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Experimental Leptospirosis. VI. Histopathology of the Bovine Placenta in *Leptospira Pomona* Infections

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ABORTIONS, usually occurring during the last trimester of pregnancy, have been reported as a manifestation of bovine leptospirosis.[3-6, 8-10, 13, 14] In the United States, bovine leptospiral abortions are due almost exclusively to *Leptospira pomona* infections, and abortions may be the only manifestation of the disease. The pathogenesis of the abortions has not been defined.

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Te Punga and Bishop[15] have postulated three possible causes for leptospiral abortions: (1) pyrexia and systemic reaction resulting in abortion; (2) interrupted transfer of metabolites, due to localized lesions in the maternal-fetal cotyledonary junction, with subsequent fetal death; and (3) actual invasion of the fetus by the leptospiras, with death and expulsion following active fetal infection.

Ferguson *et al.*[5] proposed as a possible cause the release of a toxic material from the leptospiras which is able to cross the placental barrier, where it is presumed to destroy the erythrocytes of the

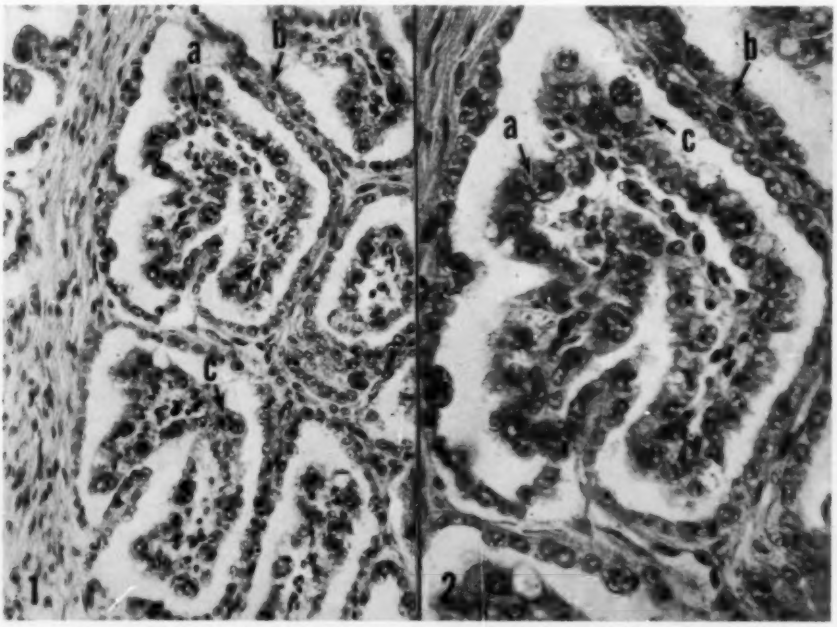

Fig. 1—Apparently normal bovine cotyledon, showing (a) fetal villus, (b) maternal epithelium with little connective tissue stroma, and (c) diplokaryocyte. Separation of fetal and maternal tissues is an artifact. x 175.

Fig. 2—Apparently normal bovine cotyledon, showing (a) fetal villus, (b) maternal epithelium, and (c) diplokaryocyte. x 325.

fetus. Alteration of the epithelium of the maternal crypts, the site of production of the hormones of pregnancy in some species, could result in hormonal inbalance and abortion.

Abortions occur two to three weeks after the acute systemic phase of leptospirosis and after the expelled fetus probably has been dead 24 to 48 hours.[3, 5, 9] Attempts to isolate leptospiras from fetal tissues, fetal fluids, or the placentas generally have been unsuccessful, indicating that *L. pomona* does not cross the placental barrier in pregnant cattle.[3–5, 10, 11] The report of Podgwaite *et al.*[12] of isolation of *L. pomona* from 3 aborted bovine fetuses has been reviewed by Ferguson *et al.*[5] The demonstration of Leptospira-like bodies in fetal material by silver staining techniques has been reported but not substantiated by bacteriological evidence.[3, 15] Leptospiras may be unable to survive in the environment of the autolyzing fetus. However, attempts to isolate the agent from living bovine fetuses, removed at slaughter at various intervals following maternal infection, have been unsuccessful.[10, 11]

Histopathological examination of the cotyledons of pregnant heifers, killed at selected intervals following infection, was undertaken to determine if alterations in the maternal-fetal placental relationships had occurred.

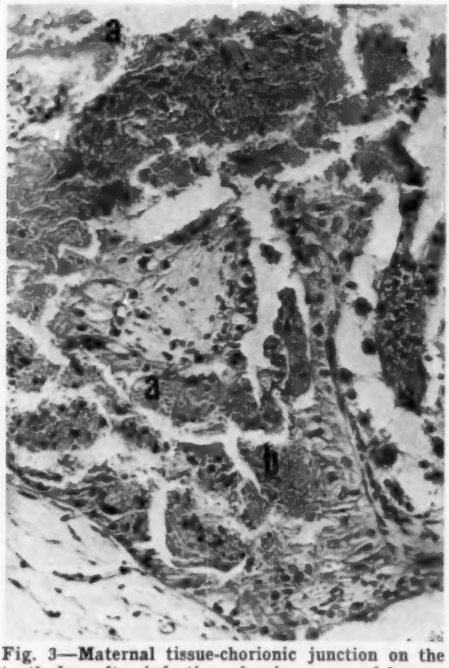

Fig. 3—Maternal tissue-chorionic junction on the tenth day after infection, showing areas of hemorrhage (a) and hemolysis (b). x 300.

MATERIALS AND METHODS

The bacteriological and serological results of experimental infection of 7 pregnant heifers with *L. pomona* have been reported.[10] Heifers 1 through 5 were infected during the sixth and seventh months and heifers 6 and 7 were infected during the third and fourth months of pregnancy. At necropsy, both fetal and maternal tissues were observed for macroscopic changes.

The maternal tissues collected for examination were: kidney, liver, cotyledons, udder, and lymph nodes; the fetal tissues were: liver, spleen, kidney, lung, and fetal membranes. All materials for histopathological examination were fixed in 10 per cent buffered formalin solution. Representative blocks of tissue were embedded in paraffin and sectioned at 7 μ. The sections were stained with hematoxylin and eosin and the Warthin-Starry silver impregnation technique.[1]

RESULTS

The clinical manifestations of the infection were mild. During the acute phase of the disease, the experimental heifers had rectal temperatures of 103.2 to 104.4 F. Six of the 7 showed anorexia, tachycardia, and depression. Hemoglobinuria, icterus, or diarrhea were not observed. The animals were killed ten to 46 days after exposure. The architecture of the cotyledon of a noninfected cow is shown (fig. 1, 2).

Heifer 5 was killed on postinfection day 10, during leptospiremia. The fetus was approximately 7 months old, viable, and appeared normal at necropsy. Significant microscopic lesions were not observed in the fetal tissues. Grossly, the uterus and cotyledons appeared normal. Microscopically, some cotyledons showed large areas of hemorrhage, with erythrocytic hemolysis and hemosiderosis at the junction of the allantochorion and the maternal tissue (fig. 3). The relationship of the chorionic villi to the maternal crypts was normal. Some vacuolation of the chorionic cells was observed. Similar changes occurred in the cotyledons of 1 heifer killed 12 days after infection.

Heifer 2 was killed 25 days after infection. The 6-month-old fetus was viable and apparently normal, as were the uterus and cotyledons. Areas of hemorrhage at the junction of the chorion and maternal tissue were observed microscopically. In the deeper portions of the cotyledons, there were some hemorrhagic areas and degeneration of the fetal villi, characterized by vacuolization (fig. 4, 5). Mitotic figures occasionally were seen in the epithelium.

The maternal crypts showed increased fibrous connective tissue.

A viable 4-month-old fetus was obtained from heifer 6 at necropsy on postexposure day 28. The only gross lesion observed was one small gray-white area in the dam's renal cortex. The most pronounced histopathological changes involved the chorionic villi (fig. 6), some of which showed vacuolar degeneration. Cellular detail was lacking in these villi and pyknosis was pronounced. The epithelial lining of the maternal crypts was disrupted in some areas and showed pyknosis. This would indicate that a degenerative process had resulted in a loss of fetal-maternal relationship in these areas. Increased amounts of connective tissue were present in the stroma of the maternal crypts.

Heifer 7 was killed 46 days postinfection. The 5-month-old fetus was viable, and no gross lesions were observed in the fetus, fetal membranes, or uterus. Numerous grayish white foci were evident in the maternal kidneys and extended through the cortex into the medulla. Microscopically, the maternal placentas (cotyledons) were found to have many empty crypts, some nearly devoid of an epithelial lining. Adjacent crypts contained cellular debris or degenerating fetal villi (fig. 7, 8). There was an increase of fibrous connective tissue in the maternal crypts.

The 6-month-old fetus of heifer 4 was alive 47 days after infection. Gross lesions were limited to the maternal kidneys. The microscopic lesions of the cotyledons resembled those of heifer 7. These changes included increased amounts of connective tissue in the maternal cotyledons, degenerated chorionic villi, and separation of the villi from the crypts.

Heifer 3 aborted 29 days after experimental infection, or 22 days after showing a systemic reaction to the infection. The 7-month-old fetus appeared to have been dead for 12 to 24 hours at the time of abortion, and mild autolysis was evident. The fetal organs appeared edematous. The pleural and peritoneal cavities of the fetus con-

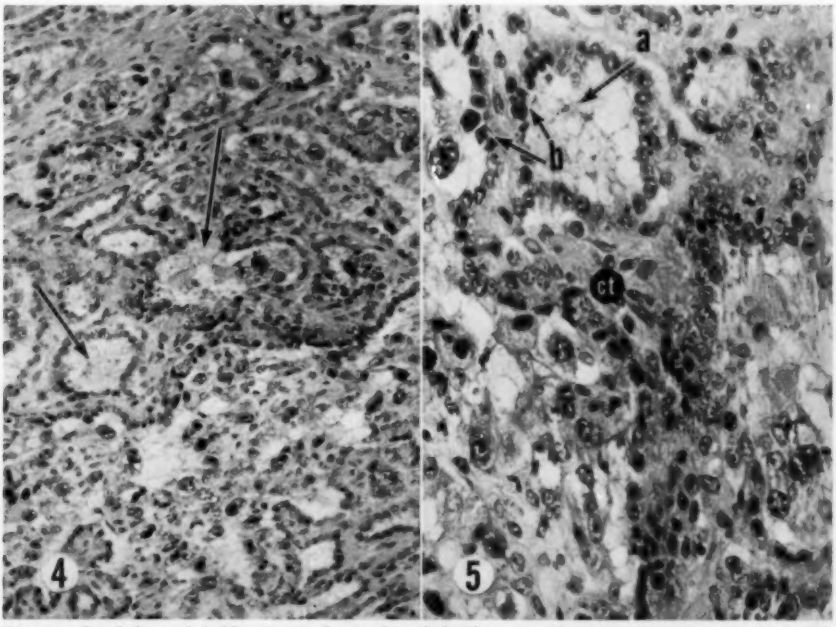

Fig. 4—Cotyledon of heifer 2, 25 days after infection, showing vacuolization of fetal villi (arrows). x 300.

Fig. 5—Cotyledon, 25 days after infection, showing increased fibrous connective tissue and vacuolization of the fetal villi: (a) vacuolar fetal villus; (b) pyknotic nuclei in the maternal epithelium; and (c) maternal connective tissue. x 135.

tained increased amounts of a serosanguineous fluid, as did the pericardal sac. The fetal membranes were retained. The heifer was killed 24 hours after abortion. Microscopically, the uterus was edematous and the bases of the maternal crypts were congested. The architecture of the cotyledons was abnormal, and the connective tissue proliferation obliterated most of the maternal crypts (fig. 9). The crypts were devoid of the cuboidal epithelial lining. The few retained fetal villi were markedly necrotic (fig. 10). Leptospiras were isolated from the maternal kidneys, but not from the fetus or cotyledons.

The gross and microscopic lesions in the maternal kidneys were similar to those reported previously.[6] Macroscopically, the capsules of the kidneys stripped with little difficulty. The cortex had numerous grayish white foci which measured up to 5 mm. in diameter and, in some instances, the lesions involved the medulla and hilus. Microscopically, these lesions consisted of a marked infiltration of lymphocytes, a few plasma cells, and macrophages. In the same areas, some tubules showed atrophy and necrosis. About 47 days postinfection, fibrous connective tissue proliferation caused a thickening of some of Bowman's capsules of the renal corpuscles. Increased fibrous connective tissue in the intertubular areas led to some tubular atrophy and necrosis.

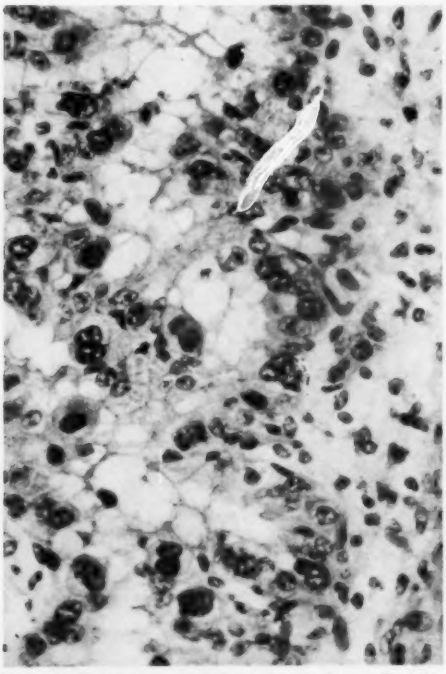

Fig. 6—Cotyledon of heifer 6, 28 days after infection, showing vacuolation of fetal villi. x 300.

Terminal serums from the heifers gave agglutination-lysis reactions at dilutions of 1:1,000 to 1:1,000,000. Leptospiras were not isolated from any of the fetuses, and they were not demonstrated by the silver staining technique applied to fetal and placental tissues.

DISCUSSION

One of 7 heifers infected experimentally with *L. pomona* aborted 22 days after the acute phase of the disease. The aborted 7-month-old fetus appeared to have been dead 12 to 24 hours, and only slight autolysis was observed. The other 6 heifers were killed ten to 47 days after infection. Their 4- to 7-month-old fetuses were viable at necropsy, and appeared normal macroscopically, as did the uteri and fetal membranes.

Microscopic changes, progressing from postinfection days 10 to 47, were found in the cotyledons, and were most marked in the cotyledons of the heifer which aborted. At day 10, areas of hemorrhage and hemolysis appeared at the chorionic-maternal tissue junction (fig. 3). By days 25 to 28, the chorionic villi had a vacuolar appearance, pyknosis and karyorrhexis of the epithelium lining the maternal crypts was evident, and proliferation of the interstitial connective tissue between the crypts was apparent (fig. 4–6).

By days 46 and 47, there was an increase in fibrous connective tissue in the maternal carunele, a loss of epithelium lining the crypts, and an absence of fetal villi (fig. 7, 8). The normal architecture of the cotyledons of the heifer which aborted was almost completely masked by the increased amounts of connective tissue (fig. 9, 10). Few necrotic villi were present, and a morphological relationship between the fetal and maternal tissues which would permit normal function appeared to be almost completely lacking.

The pathogenesis of bovine leptospiral abortions apparently can be explained, therefore, by the alterations in the intimate relationships between the fetal and maternal systems that are necessary to support

fetal development. The microscopic lesions demonstrate a series of changes of increasing severity from the time of the active infection in the dam until abortion occurs. These alterations in the placentas can interfere with the transfer of essential materials across the placental barrier, resulting in fetal inanition and death. The dead fetus becomes a foreign body and is expelled.

Direct infection of the fetus by *L. pomona* lacks unequivocal bacteriological proof at this time. The inability to demonstrate leptospiras in any of the 7 fetuses is in agreement with most reports.[3-5,11] Six fetuses were viable at the time materials were taken for bacteriological examination. Therefore, destruction of leptospiras in the environment of an autolyzing fetus could not account for the negative bacteriological results. Isolations were accomplished from the cotyledons of 1 heifer during the leptospiremic phase of the disease, but not from her fetus. This indicates that the organisms approached but failed to cross the placental barrier during the leptospiremia of the dam.

Similar results were obtained by Linquist *et al.*[7] with experimentally infected pregnant ewes. Leptospiras were isolated from the cotyledons of 1 ewe during leptospiremia. The fetal organs, blood, and fluids from this ewe and all ewes killed during the experiment were bacteriologically negative.

Positive results must be obtained by accepted bacteriological methods to confirm the presence of *L. pomona* in fetal tissues. An inoculum composed of a 10.0 per cent emulsion of 200 mg. of tissue in 0.85 per cent sterile saline solution containing ten or fewer leptospiras will infect guinea pigs or hamsters.[2] If only a few leptospiras are present in tissues, they may not be recognized by dark-field examination or staining techniques, but can be recovered by guinea pig inoculation.[16] Silver impregnation techniques can be valuable in determining the presence of leptospiras in tissues, but can not be considered a definitive diagnostic means for *L. pomona* infections unless the tissues are found to be bacteriologically positive for leptospiras.

Leptospira sejroe, Leptospira ictero-

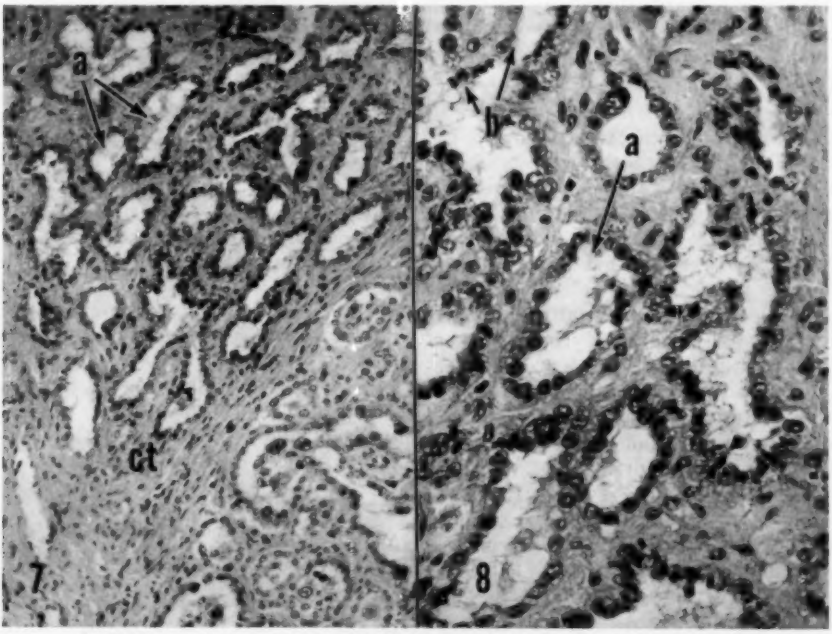

Fig. 7—Cotyledon of heifer 7, 46 days after infection, showing marked proliferation of connective tissue stroma of the cotyledon (ct), and debris in crypts (a). x 135.

Fig. 8—Cotyledon of heifer 7, showing empty crypts (a) with pyknotic nuclei (b), and lack of continuity in the maternal epithelium. x 300.

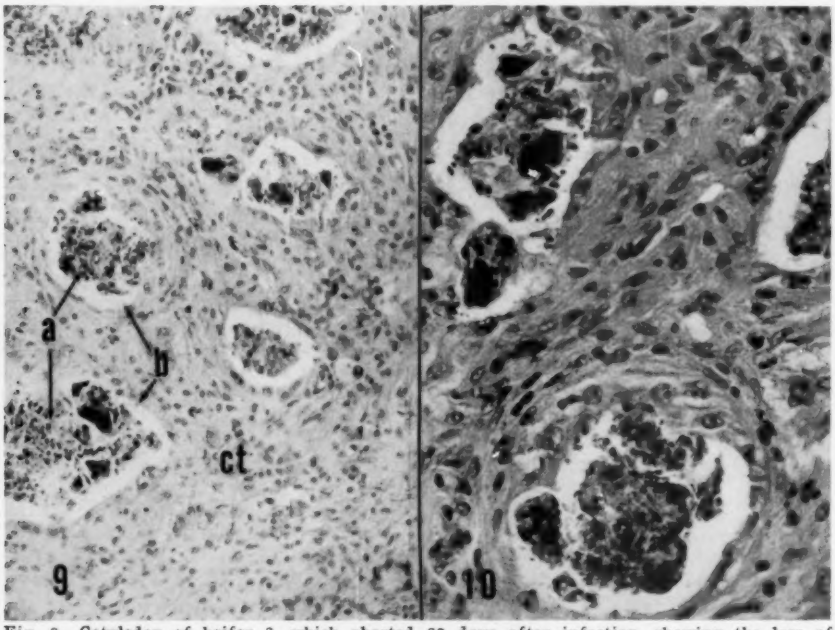

Fig. 9—Cotyledon of heifer 3, which aborted 29 days after infection, showing the loss of the normal placental architecture: (a) necrotic remnants of fetal villi; (b) absence of maternal epithelium; and (ct) maternal interstitial fibrous connective tissue. x 135.

Fig. 10—Higher magnification of figure 9. x 300.

haemorrhagiae, and *Leptospira canicola* infections may result in the invasion of the bovine fetus. The leptospiras observed by some in fetal tissues stained by silver impregnation techniques may actually be one of these three serotypes and not *L. pomona*. Clarification of the entire matter awaits experimental proof and should not be based on conclusions drawn from naturally occurring infections. Various argyrophilic tissue components can interfere with proper interpretation of silver-stained histological preparations by untrained personnel.

Bovine leptospiral abortions occur three to four weeks after infection of the dam. The fetuses apparently die shortly before abortion. If fetal death were associated with the systemic reaction of the dam, it is questionable that abortion would be delayed up to four weeks.

Fetal hematological determinations were not undertaken, but intact erythrocytes were present in the histological preparations of the fetal tissues.

CONCLUSIONS

One of 7 pregnant heifers infected with *Leptospira pomona* aborted a 7-month-old fetus 29 days after infection. Leptospiras were not isolated from the aborted fetus or from any of the 6 fetuses, 4 to 7 months old, which were viable at the time the dams were killed.

Direct infection of the bovine fetus did not appear to be responsible for the abortions under these conditions.

A series of histopathological changes was found in the cotyledons, which could interfere with the development of the fetus and result in fetal death and abortion.

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Lungworm Infections in Calves Produced by Subcutaneous Injections of Larvae

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EXPERIMENTAL infections of cattle with the lungworm, *Dictyocaulus viviparus* (Bloch), have been established by the oral administration of third-stage larvae.[1-4, 6-9] Clinical signs produced by lungworm infections generally appear seven to 15 days after exposure to infective larvae.[2, 5, 7, 8] The prepatent period is between 21 and 33 days.[2, 4, 6-10]

This experiment was designed to determine whether lungworm larvae injected subcutaneously would produce infection and, if so, whether the course of the disease would differ from infections produced by the oral administration of larvae.

MATERIALS AND METHODS

Cross-bred dairy calves used in these experiments were allowed to nurse for three to seven days. They were then placed in individual pens and fed milk substitute for 30 days, followed by concentrate, hay, and minerals. All calves were raised lungworm-free until they were 3 to 5 months old, when the principals were given varying quantities of viable lungworm larvae subcutaneously. Four calves raised under conditions similar to the principals were given no larvae and served as controls.

The lungworm larvae used in these experiments were obtained from the feces of experimentally and naturally infected calves. The larvae (except those used in calf 22) were collected, washed with tap water, placed in Petri dishes containing animal charcoal and water, and allowed to develop to the third stage. First-stage larvae, 1 and 2 days old, were given to calf 22. The 2-day-old larvae were refrigerated at 5 C. immediately after they were recovered from the Baermann funnel, in order to arrest their development. Before being given to the calves, the infective-stage larvae were washed and the number living was calculated from aliquot samples of the total suspension. All active larvae and those having a transparent, glistening appearance were considered viable. The larvae were concentrated by centrifugation at approximately 2,400 r.p.m. for two minutes and suspended in 5 ml. of water. To each milliliter of this suspension was added 20 mg. of streptomycin sulfate, U.S.P.; 20,000 units of penicillin G potassium, U.S.P.; and 10 units of nystatin (Mycostatin*). The mixture was shaken frequently during 20 to 40 minutes, then centrifuged again and the supernatant fluid discarded. The larvae were washed twice with 5-ml. portions of sterile water, resuspended in 5 ml. of sterile water, and immediately injected subcutaneously.

Fecal examinations were conducted two to 20 days after the injection of larvae and at intervals of one to seven days during the patent period of infection, using a Baermann apparatus.

RESULTS

Calf 22 was given 500,000, 1,851,000, and 2,038,000 first-stage lungworm larvae subcutaneously, in the cervical region, at seven-day intervals. Beginning 27 days after the initial injection and continuing for 41 days, lungworm larvae were recovered from the feces. The peak output was 19 larvae per gram of feces. The calf coughed occasionally during the latter part of the patent period. A control calf remained free of lungworms during this time.

Calf 23 was given 154,500 third-stage lungworm larvae subcutaneously in the right cervical region and, eight days later, 175,000 third-stage larvae in the left cervical region. An increased rate of respiration was observed 17 days after the initial injection of larvae; severe respiratory distress developed by the twenty-sixth day, and the calf died on the thirty-first day after the initial injection. Larvae were never removed from the scanty feces of this animal; however, at necropsy, 10,993 adult and immature lungworms were recovered from its lungs. There were no gross pathological changes at the site of larval injection. A control calf remained free of lungworms during this time.

Calf 25 was given three injections of 86,000, 68,000, and 6,000 third-stage lung-

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* Mycostatin is produced by E. R. Squibb and Sons, 745 Fifth Ave., New York 22, N.Y.

worm larvae subcutaneously, in the cervical region, at intervals of eight and seven days, respectively. Respiratory distress was first observed on the thirteenth day, and larvae were recovered from the feces of this calf on the twenty-fifth day after the initial injection. A peak larval count of 989 per gram of feces was recorded on the thirty-sixth day. This animal died on the eighty-second day after the first injection of larvae and, at necropsy, 156 adult lungworms were recovered from the extensively consolidated lungs. A control calf remained free of lungworms during this time.

Calf 31 was given a total of 87,300 third-stage larvae in four subcutaneous injections in the right and left flanks over an eight-day period. Respiratory difficulty first was observed on the seventeenth day after the initial injection. The prepatent period was 26 days, and the peak larval count was 297 per gram of feces. The calf recovered and was not euthanatized. A control calf remained free of lungworms during this time.

Calf 32, given one injection of 94,000 third-stage larvae subcutaneously in the gluteal region, showed respiratory difficulty on the fifteenth day, and larvae first were recovered in the faces on the twenty-ninth day postinjection. This calf recovered and was not euthanatized.

DISCUSSION

The results of this investigation show that lungworm larvae are capable of causing characteristic infections when given subcutaneously. This suggests that, if larvae gain access to the subcutaneous tissues through wounds or insect bites, they may produce infection.

Third-stage larvae were better able to produce infections than were 1- and 2-day-old first-stage larvae. A mild infection resulted in calf 22 from a total injection of first-stage larvae which was 27 times greater than that necessary to produce death in calf 25 with third-stage larvae. That it was possible to produce infection with first-stage larvae probably was due to the fact that some of the injected larvae were deposited in a site beneath the skin that was favorable for their development to the third stage.

Further experiments are necessary to determine whether lungworm larvae are capable of penetrating the unbroken skin or of entering the host through skin abrasions.

SUMMARY AND CONCLUSIONS

1) Viable lungworm larvae administered subcutaneously in the cervical, flank, and gluteal regions produced lungworm infections in calves.

2) Signs of lungworm infection first appeared 13 to 17 days after the initial injection of larvae, with prepatent periods of 24 to 28 days.

3) These infections appeared similar, clinically, to infections produced by the oral administration of larvae to calves.

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Studies on the Oral Administration of Sulfaquinoxaline in Dairy Cattle—Pharmacology and Pathology

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SULFAQUINOXALINE (2-sulfanilamidoquinoxaline), synthesized by Weijlard *et al.*,[27] in 1944, was first shown to have antibacterial activity by Smith and Robinson.[23] Its unusual property of producing prolonged blood levels also was pointed out by these authors. Seeler *et al.*[21] found that sulfaquinoxaline remained in the blood for days and that it possessed some activity against certain species of malarial parasites. These workers also observed that, during its excretion, crystals of the metabolized 3-hydroxy- derivative of the drug could be found in the distal convoluted tubules and collecting tubules of the kidney, probably because of its relative insolubility.

In the report of the School of Veterinary Medicine, Michigan State College, for 1945, sulfaquinoxaline was shown to have both prophylactic and therapeutic activity against *Eimeria tenella*. This observation soon was confirmed by Delaplane *et al.*,[7] Grumbles *et al.*,[13, 14] and others,[3, 5, 18] and established sulfaquinoxaline as an efficacious coccidiostatic agent in chickens and turkeys. It is apparent that the practical chemotherapeutic properties of the drug were established before many reports of its pharmacological activity in poultry appeared in print. However, since 1950, papers dealing with the effects of sulfaquinoxaline on the physiology of fowl have been published by Schlenker and Simmons,[20] Bankowski and Johnson,[2] Cuckler and Ott,[6] and others.[19, 21, 26] Sulfaquinoxaline continues to have an important role as a coccidiostatic and antibacterial agent in poultry practice.

The success of the drug in poultry disorders naturally caused it to be considered for wider use in infectious diseases of mammals. Early investigators[4] reported that large single doses of sulfaquinoxaline (up to 400 mg./kg. of body weight) could be given to healthy lambs without untoward effects. The clinical efficacy of sulfaquinoxaline in coccidiosis and other infections of large animals has been reported by Dunlap,[9] Frank,[12] and others.[1, 10, 11, 17]

It is established that all the readily absorbed sulfonamides have definite toxicity for animals when used in large doses for prolonged periods. Sulfaquinoxaline is no exception. Stevens *et al.*,[24] Seeler *et al.*,[21] and Cameron and McOmie[4] have reported the presence of crystals of metabolic byproducts of sulfaquinoxaline in animals given large doses. The rapid development of hypoprothrombinemia in rats and dogs has been observed by Seeler *et al.*[21] and Mushett and Seeler,[16] while a "drug-shock" syndrome following a large dose of sulfaquinoxaline in a cow was reported by Jones *et al.*[15]

Since the use of sulfaquinoxaline in certain infectious diseases of cattle seemed to offer promise, it was important that the toxicity of this drug for bovine animals be established. The pharmacological studies described in this report were undertaken to determine the maximum tolerated dose of sulfaquinoxaline in dairy cattle. Most of the work was done with the soluble material administered by mouth, since it was realized early in the study that solutions given orally elicited a more marked clinical reaction than the insoluble material given by capsule. The soluble material used was, in most cases, 50 per cent weight in volume concentration (w/v) sulfaquinoxaline in sodium hydroxide and potassium hydroxide solution. The insoluble material used was pure sulfaquinoxaline powder.

EXPERIMENTAL PROCEDURE

The 59 cows used for these studies were not selected for uniformity in breed, size, or age. They were in various stages of lactation and many, if not all, were culls. In a few instances, therefore, unapparent organic and pathological aberrations were present which may have influenced the degree of sulfaquinoxaline reaction.

Plasma level assays using soluble sulfaquinoxaline given as a drench indicated that the observed onset of the reaction corresponded to the peak plasma levels achieved (between 3 and 5 hours). However, animals given insoluble sulfaquinoxaline in capsules in doses as high as 530 mg. per kilogram * had higher plasma levels before showing

From the Merck Sharp and Dohme Research Laboratories, Rahway, N.J.

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* All doses, originally given in grains, have been recalculated to the nearest 5.0 mg. Hence, 65.0 mg. per kilogram approximates 0.5 gr. per pound.

TABLE 1—The Relationship of Various Doses of Soluble Sulfaquinoxaline Given via Drench to Signs of Toxicosis

Animal (No.)	Dose (mg./kg.)	No. doses	Signs of intoxication*	Day noticed	Time of slaughter
1	265	1	1	1	72 hours
835	265	3	1, 2	1	4th day
836	265	3	1, 2, 3, 4	1	3rd day
972	265	4	1, 2, 4	2	5th day
149	265	4	1, 4	2	5th day
150	265	2	1, 5	2	3rd day
2	130	1	....	..	3rd day
839	130	3	2	1	4th day
841	130	3	2	1	4th day
971	130	4	4	5	5th day
151	130	2	1, 5	2	3rd day
152	130	4	....	..	5th day
153	130	4	....	..	5th day
405	130	3	1, 2, 5	1	4th day
3	65	1	....	..	72 hours
844	65	3	....	..	4th day
845	65	3	....	..	4th day
973	65	4	1, 4	4	5th day
339	65	4	1	4	5th day
147	65	4	....	..	5th day
146	65	4	....	..	5th day
148	65	4	....	..	5th day
842	30	3	2	1	4th day
843	30	3	....	..	4th day
970	30	4	....	..	5th day
4	15	1	....	..	72 hours
838	15	3	....	..	4th day
840	15	3	....	..	4th day

* 1 = ataxia and muscular weakness; 2 = inappetence; 3 = hematuria; 4 = gross kidney crystals; and 5 = renal edema, gross (last two indicate necropsy findings).

any reaction. It would appear from a comparison of these two groups of animals that the rapidity at which peak plasma levels are reached influences the degree of drug reaction (graphs 1, 2). A partial explanation for this difference in the speed at which peak plasma levels are obtained may lie in the fact that the capsuled material retains its homogeneity in the rumen for six to 12 hours and, thus, is less readily available for absorption, whereas the soluble material administered by drench is dispersed rapidly throughout the rumen. Veterinary surgeons often have found relatively intact boluses of medication during the course of rumenotomies performed eight hours after the last medication, thus emphasizing the need for readily soluble or dispersible pharmaceutical preparations for oral use in the ruminant if rapid systemic effects are desired.

Seven cows were used for studies comparing four sulfonamides: sulfaquinoxaline, sulfamethazine, sulfathiazole, and sulfabromomethazine. Cow 149, seven months' pregnant, was not disturbed by soluble sulfaquinoxaline medication at doses of 265 mg. per kilogram daily for four days, despite the fact that she did show a clinical reaction (staggering) and had sulfaquinoxaline crystals in both kidneys at necropsy.

Experiments with 59 cows are classified into four categories: (1) the effects of soluble sulfaquinoxaline given to cows by drench or drinking water; (2) the effects of insoluble sulfaquinoxaline given to cattle by capsule; (3) palatability to cows of sulfaquinoxaline-medicated water; and (4) comparative toxicity to cows of four sulfonamides.

RESULTS AND OBSERVATIONS

Category 1.—Soluble sulfaquinoxaline was given to 28 cows as a drench at dosage levels of 15 to 265 mg. per kilogram daily,

TABLE 2—The Relationship of Various Doses of Insoluble Sulfaquinoxaline Given via Capsule to Signs of Toxicosis

Animal (No.)	Dose (mg./kg.)	No. doses	Signs of intoxication*	Day noticed	Time of slaughter
847	530	6	1, 2, 3, 4	2 (slight inappetence)	7th day
713	530	6	1, 2, 4	2	7th day
5	265	8	1, 2, 3, 4	1	9th day
791	265	6	2, 4	2	7th day
846	265	6	4	7	7th day
6	130	7	4	8	8th day
881	130	6	..	..	7th day
7	65	8	†	..	9th day

* In these animals, 1 signifies trembling and slight weakness (*see* footnote for table 1). † Cow 7 showed chronic pyelonephritis on necropsy.

TABLE 3—Individual Water Intake (Nonmedicated) of Animals Given Sulfaquinoxaline by Drench

Animal (No.)	Dose (mg./kg./day by drench)	Intake (gal./cow) Days 1	2	3
835	265	4	4.0	9.5
836	265	8	1.0	Killed
839	130	3	4.0	6.5
841	130	5	6.0	8.0
844	65	6	4.0	7.0
845	65	10	4.0	11.0
842	30	8	4.0	10.0
843	30	5	6.0	9.0
838	15	3	10.0	5.0
840	15	4	6.5	7.0

in single or divided doses, for one to four days (table 1). Of these 28 cows, 15 also were used for palatability studies with sulfaquinoxaline-medicated water simultaneously with the drenching with sulfaquinoxaline (tables 5, 6). Doses given by drench in the 28 animals were estimated according to medicated water intake to insure total daily medication with the prescribed dosage level.

The data show that soluble sulfaquinoxaline is toxic at daily dosage levels of 265 mg. per kilogram. At levels of 130 mg. per kilogram, ataxia was noticed in 3 of 8 animals after one to three daily doses. It appears from these observations that doses of soluble sulfaquinoxaline greater than 65 mg. per kilogram should not be used.

Most of the animals were slaughtered within 24 hours after cessation of medication, so that there was little opportunity to observe whether signs of intoxication would disappear after the drug was withdrawn.

Category 2.—Eight cows were given insoluble sulfaquinoxaline in capsules in single daily doses of 65 mg. per kilogram to 530 mg. per kilogram for one to four days (table 2.)

On the basis of this experiment, it appears that insoluble sulfaquinoxaline given by capsule is not clinically toxic at dosage levels up to 130 mg. per kilogram for six days. However, 1 of 2 experimental animals given 130 mg. per kilogram daily for six to seven days had deposits of sulfaquinoxaline crystals in both kidneys. Plasma levels obtained in the animals (table 2) are shown graphically (graph 2) and should be compared with plasma levels obtained following the use of soluble material in 3 animals given 265, 130, and 65 mg. per kilogram, respectively (graph 1). Animals 5, 6, and 7 are not represented in the graphs, since blood samples were drawn only once during the course of medication in this group.

Category 3.—Ten cows drank 0.025 per cent sulfaquinoxaline-medicated water (1.0 Gm./gal.) ad libitum for four days. A fresh supply of medicated water was provided each day. The average daily consumption of medicated water in gallons per cow for each of four consecutive days was 0.5, 3.3, 4.0, and 4.2 gal.

These animals were kept together in a pen and only the average intake for each animal could be measured. On the first day, water consumption was low but, on subsequent days of the experiment, water intake increased daily. However, an average nonlactating dairy cow may be expected to consume between 8 and 12 gal. of water daily. The low water intake in all these experiments could be attributed to several factors, such as: (1) unfamiliarity with drinking from a trough, (2) psychic stress of new surroundings, (3) polled cows being harassed by horned animals, and (4) the bitter taste of the sulfaquinoxaline preparation.

The water intake of 10 animals given sulfaquinoxaline by drench was measured over a three-day period (table 3). The animals were watered individually twice daily from a 5-gal. pail and, in each instance, finished drinking in less than five minutes.

There seems to be no significant differ-

TABLE 4—Palatability of Water Medicated with Sulfaquinoxaline at Different Levels

Animal (No.)	Dose (mg./kg./day by drench)	Intake (gal./cow) Amount sulfaquinoxaline in water (Gm.) 2.0	None	1.0	None	0.5	None
		Day 2*		Day 3		Day 4	
972	265	0	7	1	3	0	3
971	130	0	5	0	4	3	2
973	65	0	10	0	8	11	0
339	65	0	6	0	0	1	1
970	30	0	3	0	5	7	0

* No water was offered on day 1. Animals were started on the experiment at 3:00 p.m. and had free access to unmedicated water.

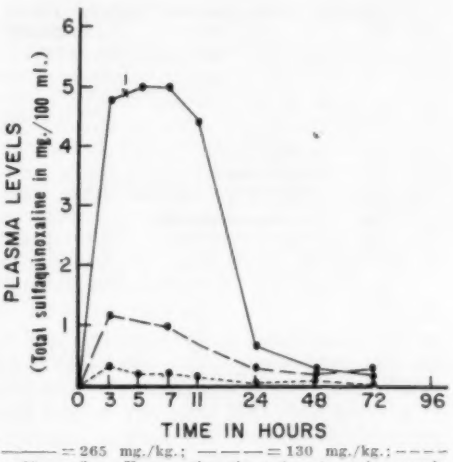
ence in water intake between animals drenched with either high or low doses of sulfaquinoxaline.

Five cows were given sulfaquinoxaline-medicated water at levels of 0.5 to 2.0 Gm. per gallon to determine palatability (table 4). At the same time, they were being drenched with soluble sulfaquinoxaline to insure a predetermined daily intake. Immediately after the medicated water was removed, untreated water was offered to these cows for five to ten minutes.

It seems apparent that water containing more than 0.5 Gm. of sulfaquinoxaline per gallon was unpalatable. It has been reported, however, that sulfaquinoxaline-medicated drinking water (1 Gm./gal.) is palatable for both beef cattle [17] and feedlot lambs.[11] Water containing 0.5 Gm. of sulfaquinoxaline per gallon was consumed by 4 of 5 animals, but 3 of the 5 consumed an additional quantity of untreated water. This would indicate that even the lowest concentration of sulfaquinoxaline appreciably reduced palatability.

Nine animals were given sulfaquinoxaline at doses of 0.25 Gm. per gallon of drinking water and later were offered untreated water. The daily medicated and untreated water intake was measured (table 5).

These data indicate that 0.25 Gm. of sulfaquinoxaline per gallon of water is acceptable to dairy cows. The average medicated water intake of 6 animals on the last day was 5.07 gal. In the previous group of animals (table 4), the average consumption on the last day of the experiment was 8.10 gal. The difference of 3.06 gal. may be accounted for by unpalatability of the sulfaquinoxaline-medicated water or by the several variables mentioned earlier. To determine palatability more precisely, it would be desirable to use larger groups of animals under more normal environmental conditions.



——— = 265 mg./kg.; — — — = 130 mg./kg.; - - - - = 65 mg./kg.; X = reaction time; 1 = staggering, weakness, tremors.

Graph 1—Plasma levels and signs of toxicosis in cows given soluble sulfaquinoxaline orally in single doses.

Category 4.—The comparative toxicity of the sodium salts of sulfamethazine, sulfathiazole, sulfabromomethazine,* and sulfaquinoxaline, as well as palatability of water medicated with each of these drugs at doses of 0.25 Gm. per gallon was studied in four groups of 2 cows each, except that only 1 cow was used in the sulfaquinoxaline experiment (table 6). Each cow was given a total daily dose of 130 mg. of its respective sulfonamide per kilogram of body weight. A portion of the medication was offered

* Experimental compound, supplied by the Merck Sharp and Dohme Research Laboratories, Rahway, N.J.

TABLE 5—Water Consumption of Animals Given Sulfaquinoxaline-Medicated Water (0.25 Gm./gal. for 3 Days)

Animal (No.)	Dose (mg./kg./day by drench)	Intake (gal./cow)					
		Day 2*		Day 3		Day 4	
		Medicated	Nonmedicated	Medicated	Nonmedicated	Medicated	Nonmedicated
149	265	3.5	0	4	0	4.0	0
150	265	2.5	2	Killed	..	....	..
151	130	7.0	0	Killed	..	....	..
152	130	3.0	2	8	0	6.0	0
153	130	8.0	0	10	0	6.0	0
405	130	5.5	0	0	9	0.0	0
147	65	6.0	0	11	0	7.5	0
146	65	4.5	0	7	0	8.0	0
148	65	8.0	0	5	0	4.0	0

* No water was offered on day 1, since animals had had free access to unmedicated water immediately before experiment.

TABLE 6—Comparative Toxicity of Four Sulfonamides, Judged by Water Consumption (0.25 Gm. of Drug/gal.)

Animal (No.)	Drug*	Intake (gal./cow) Day 1 Medicated	Day 1 Nonmedicated	Day 2 Medicated	Day 2 Nonmedicated	Day 3 Medicated	Day 3 Nonmedicated
409	Sulfamethazine	3.5	0	5	0	4	6
979	Sulfamethazine	6.0	0	0	0	2	4
47	Sulfathiazole	5.0	0	1	0	5	0
994	Sulfathiazole	5.5	0	3	0	5	3
95	Sulfabromomethazine	8.0	0	5	0	7	0
411	Sulfabromomethazine	12.0	0	2	0	7	3
405	Sulfaquinoxaline	5.5	0	0	9	0	0

* Each animal was given a total of 130 mg. per kilogram daily of the respective sulfonamide (sodium salt).

in the water twice daily (0.25 Gm./gal.); the remainder was given in two equal doses as a drench. Water consumption conformed in general with that observed in previous palatability studies. The sulfaquinoxaline-medicated animal seemed somewhat intoxicated, which undoubtedly influenced water consumption. There was evidence of staggering on the first day and some signs of incoordination throughout the experiment.

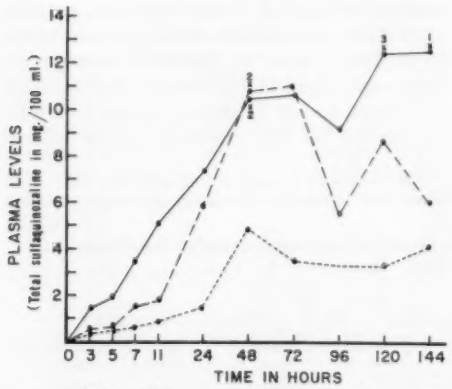
PLASMA LEVELS

A comparison of plasma levels of the four groups of animals given different sulfonamides is shown (table 7, graph 3). Sulfamethazine gave the highest plasma levels; sulfabromomethazine gave the next highest, apparently with a cumulative tendency; and sulfaquinoxaline gave the third highest level, reaching a peak of 14.66 mg./100 ml. on the third day. Although the levels obtained with sulfathiazole were low, this dose (130 mg./kg./day in 2 doses) closely approximates the reportedly effective clinical dose.

Sulfaquinoxaline is detectable in the blood four to five hours after administration of doses as low as 15 mg. per kilogram (table 8). This is of interest, as continuous medication may be desirable for the prophylaxis or treatment of a particular disease. Drug levels in the plasma four to five hours after sulfaquinoxaline administration are shown. In all cases, except for cow 405, the drug was given once daily.

The plasma drug levels indicate a tendency for sulfaquinoxaline to accumulate in the body at doses as low as 30 mg. per kilogram daily (graph 5). The distribution patterns also show the ascending plasma values after repeated daily treatment with sulfaquinoxaline.

The plasma levels of sulfaquinoxaline, as obtained in several animals after giving various doses of soluble material, are represented (graphs 1, 4). A possible explanation for the marked plasma level difference is that daily dosage (graph 4) may increase the tendency for accumulation in the body, while a single dose may be excreted rapidly. Cows given 265 mg. per kilogram or more of sulfaquinoxaline orally daily showed signs of intoxication (graphs 1, 2). There was a tendency toward accumulation for the first two to four days, after which the plasma concentrations became erratic. The data indicate that the plasma concentration at which drug accumulation levels off may be related directly to the dose.



——— = 530 mg./kg.; — — — = 265 mg./kg.; - - - - = 130 mg./kg.; X = reaction time; 1 = staggering, weakness, tremors; 2 = inappetence; 3 = hematuria.

Graph 2—Plasma levels and signs of toxicosis in cows given insoluble sulfaquinoxaline by mouth daily for six days. Two animals were used for each dosage level, except for the 130 mg. per kilogram dose, where 1 cow was used.

CLINICAL SIGNS OF TOXICOSIS

The most common signs of clinical toxicosis following the oral administration of soluble sulfaquinoxaline were ataxia and muscular weakness (table 1). This appeared in 10 of the 28 experimental cows. It occurred in all the cows given 265 mg. per kilogram of sulfaquinoxaline on the first or second day. Two of 8 animals given 130 mg. per kilogram daily showed staggering, and 3 showed slight inappetence on the first day of medication. In the group given 65 mg. per kilogram daily, 2 of 8 cows showed signs of ataxia on the fourth day. Of the 3 given 30 mg. per kilogram, 1 showed slight inappetence on the first day, eating hay normally but refusing grain. The sudden change to a new environment could have been responsible for this reaction.

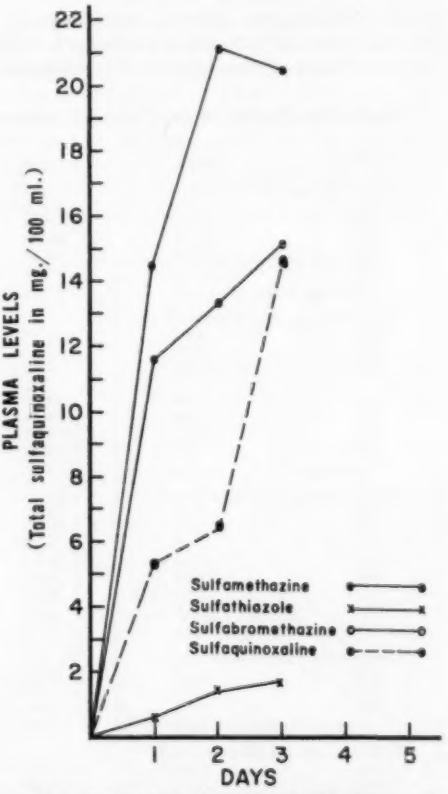
Of 8 cows given insoluble sulfaquinoxaline by capsule once daily and observed for six to eight days, the 2 given 530 mg. per kilogram daily for six days showed slight inappetence on the second day and general weakness on the last two days of medication. Ataxia was not observed. Of the 3 given 265 mg. per kilogram for six to eight days, cow 5 showed ataxia the first day, but it subsided by the ninth day in spite of continued medication. Data obtained by Stowe *et al.*[25] indicate that ataxia in cattle following the intravenous use of 65 mg. per kilogram was transient, lasting for less than 24 hours. Cow 5 also showed anorexia and hematuria, and cow 791 was anorectic on the second day. Neither of 2 animals given 130 mg. per kilogram daily for six to seven days showed any signs of intoxication. Cow 7, given 65 mg. per kilogram daily for eight days, showed no signs of intoxication, although she had severe nephritis at necropsy.

BLOOD UREA NITROGEN

Blood urea nitrogen (BUN) determinations were made at least once on blood samples of all cows (usually the last sample drawn). The average BUN level before medication in 8 cows was 3.7 mg./100 ml.

Blood urea nitrogen levels in 2 cows given insoluble sulfaquinoxaline at doses of 530 mg. per kilogram daily for six days rose from an average of 6.0 to 12.6 mg./100 ml. Although this change is not considered significant, macroscopic renal crystals were present in both cows at necropsy and hematuria was observed in 1 cow. It might be assumed from the low BUN levels that significant renal damage was not present in these animals. However, in 1 of the cows, the BUN level was as high as 40.2 mg./100 ml. after three days of treatment with 65 mg. per kilogram of soluble sulfaquinoxaline given as a drench. In all other animals given soluble medication, there was no apparent relationship between the dosage level of drug or the plasma level obtained and the BUN levels at the end of each experiment (table 9).

These findings show that BUN values increase more markedly following the use of the soluble sulfaquinoxaline than with the insoluble material. The reason for this is not clear, since there does not seem to be a



Graph 3—Comparison of plasma levels of four sulfonamides given orally. Each drug was given at levels of 130 mg. per kilogram per day in two doses for a total of five doses (72 hours.) All data represent averages from 2 cows except those for sulfaquinoxaline, which are from 1 cow.

TABLE 7—Comparative Plasma Levels of Cows Given Four Sulfonamides (Total Sulfonamide in mg./100 ml.)*

Cow (No.)	Drug	Day 1	Day 2	Day 3
409	Sulfamethazine	.....	21.25	21.25
979	Sulfamethazine	14.55	21.25	19.94
Average levels		14.55	21.25	20.59
47	Sulfathiazole	0.80	2.03	2.03
994	Sulfathiazole	0.45	0.98	1.40
Average levels		0.62	1.50	1.71
95	Sulfabromomethazine	11.77	12.71	15.70
411	Sulfabromomethazine	11.77	14.20	14.58
Average levels		11.77	13.45	15.14
405	Sulfaquinoxaline	5.47	10.13	14.66
Average levels		5.47	10.13	14.66

* Daily total dose of 130 mg. per kilogram was given in two doses by drench and in drinking water. Plasma determinations were made promptly on blood samples taken once daily, approximately four hours after the morning medication period.

linear relationship between sulfaquinoxaline values and BUN values observed with the two routes of medication. For example, an average BUN value of 17.4 mg./100 ml. was found after three days of treatment with 30.0 mg. of soluble sulfaquinoxaline per kilogram, where the highest sulfaquinoxaline plasma level was 4.0 mg./100 ml. However, in 1 animal given 130.0 mg. per kilogram for six days, the BUN value reached only 7.0 mg./100 ml. and the highest sulfaquinoxaline plasma level exceeded 5.0 mg./100 ml. An explanation for this may be the rapidity of absorption of the soluble material, resulting in greater immediate stress on renal function.

BLOOD CLOTTING

In view of reports in the literature on the effect of sulfaquinoxaline on the blood clotting mechanism,[4, 6, 20] a visual means of comparing the blood clotting time of sulfaquinoxaline-medicated animals with non-medicated animals was selected. In the procedure used, 2 cc. of blood was taken

TABLE 8—Plasma Levels (Total Sulfaquinoxaline in mg./100 ml.) of Cows on Various Dosage Levels*

Cow (No.)	Day 1	Day 2	Day 3	Day 4	Day 5
Dose—265 mg. per kilogram daily					
835	7.73	10.40	18.58	21.16	.....
836	22.06	7.99	[4.03]	.....	.....
972	12.12	15.25	16.73	[17.22]	.....
149	10.15	15.43	22.13	24.36	22.53
150	4.49	16.73	[15.25]	.....	.....
Average levels	11.31	13.16	19.14	22.76	.....
Dose—130 mg. per kilogram daily					
839	4.80	2.19	6.47	[4.89]	.....
841	6.97	7.15	10.19	[3.22]	.....
971	4.85	9.72	11.50	Hemolysis of sample	11.07
151	6.46	11.07	[3.87]	2.83	1.78
152	1.54	8.24	5.35	3.87	3.26
153	2.83	7.87	2.83	Killed	.....
405†	5.47	10.13	14.66	.....	.....
Average levels	4.70	8.05	8.50	3.30	.....
Dose—65 mg. per kilogram daily					
844	0.64	1.87	3.28	[0.81]	.....
845	2.45	7.73	9.23	[6.79]	.....
339	1.47	6.26	11.69	15.74	[15.01]
973	2.89	5.34	5.74	3.07	[2.18]
146	2.98	4.85	7.61	7.74	[4.58]
147	3.65	5.43	7.61	7.98	[1.84]
148	1.57	4.85	5.13	4.85	[3.22]
Average levels	2.23	5.33	7.18	7.87	.....
Dose—30 mg. per kilogram daily					
842	0.35	0.87	1.55	[0.29]	.....
843	3.28	3.48	4.03	[0.35]	.....
970	0.61	0.89	1.20	1.11	[0.61]
Average levels	1.41	1.74	2.26	1.11**	.....
Dose—15 mg. per kilogram daily					
838	1.09	0.87	1.32	[0.06]	.....
840	1.16	1.26	0.81	[0.06]	.....
Average levels	1.12	1.07	1.01	.....	.....

* Blood samples were taken once daily, four to five hours after medication. † This animal was given a total daily intake of 130 mg. per kilogram in two doses. ** This value was obtained in 1 animal which showed consistently lower values throughout the medication period.

Brackets [.....] indicate sample drawn on the day of slaughter. No medication was given on that day. These figures are not included in the daily averages for the group average plasma level.

from a medicated animal and placed in a standard-sized (4 by 9/16 inches) field bleeding tube. The time taken for development of a clot (table 10) was measured by gently agitating the tube once each minute.

An average clotting time for the ox, obtained by Bürker's method,[8] is listed as 6.5 minutes. At no time during the course of these experiments, using insoluble or soluble sulfaquinoxaline, were there clinical indications of increased blood clotting time.

PATHOLOGY

In view of the random selection and the variation in age and history, it was to be expected that renal pathological changes would be found with relative frequency in such a group of animals. An 11-year-old Hereford (cow 7) was found to have a chronic pyelonephritis at the time of slaughter.

To facilitate evaluation of the tissue changes seen, different groups of animals are discussed according to dosage levels, beginning with the highest levels. Soluble sulfaquinoxaline is described first, insoluble sulfaquinoxaline next and, finally, the pathological changes in the 6 animals used for a comparison of three other sulfonamides.

Observations on erythrocytes, tubular dilation, and sulfonamide crystals (microscopic and macroscopic) are considered important in this study. Renal damage, such as chronic interstitial nephritis, glomerulitis, and swelling of tubular epithelium, was seen frequently in the experimental animals but also was seen in the controls. These changes are believed to be common in the class of animals used for these studies, and undoubtedly are due to pre-experimental infections, toxins, and aging.

Although sulfaquinoxaline crystals often were observed in the kidneys at necropsy, only the cows on the highest dose showed any signs of kidney damage. Unfortunately, there was no opportunity to observe these cows for an appreciable interval after cessation of treatment to determine if post-treatment effects related to renal changes would appear. However, when one considers that similar pathological changes occurred in the kidneys of both treated and control animals, the true significance of these changes and of crystalluria seems less clear-cut than often is stated.

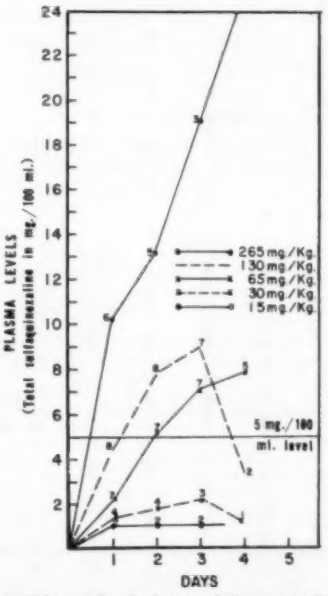
Soluble Sulfaquinoxaline (265 mg./kg.). —Moderate to severe tubular dilation of the kidneys occurred in 5 of the 7 cows which were given soluble sulfaquinoxaline for one to four days. Although gross crystals were present in the calyces of the kidneys of 3 of the 7 (cows 836, 972, 149) at slaughter, no crystals were seen microscopically. Crystals were seen microscopically but not grossly in cow 835. Hematuria was seen in cow 836 and erythrocytes were observed in the convoluted tubules in cow 150.

Soluble Sulfaquinoxaline (130 mg./kg.). —One animal (cow 971) of the group of

TABLE 9—Blood Urea Nitrogen Determinations on the Last Day of the Experiment (Average of 2 or More Animals)*

Daily dose (mg./kg.)	Blood urea nitrogen (mg./100 ml.)
Soluble sulfaquinoxaline†	
265	14.02 (av. 4 cows)
130	17.30 (av. 6 cows)
65	21.80 (av. 6 cows)
30	17.40 (av. 4 cows)
15	3.00 (av. 2 cows)
Insoluble sulfaquinoxaline**	
530	12.60 (av. 2 cows)
265	11.40 (av. 2 cows)
130	7.00 (av. 1 cow)

* The pretreatment blood urea nitrogen level for 8 animals was 3.7 mg./100 ml. † The medication period was three to four days. The drug was given daily, usually in a single dose. ** The medication period was six days.



Graph 4—Plasma levels in cows given soluble sulfaquinoxaline orally. Figures at points on graph indicate the number of animals yielding the average value.

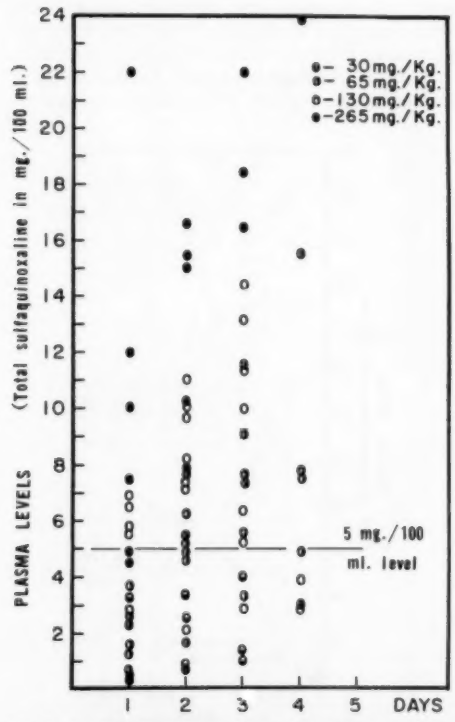
TABLE 10—Time Required for Blood Clotting in Treated Cows

Sulfaquinoxaline dose (mg./kg.)	No. daily doses	Blood clotting time*
530	6	5 minutes
530	6	8 minutes
265	6	7 minutes
265	6	6 minutes
130	6	8 minutes

* Clotting was measured 24 hours after medication period at room temperature (65 degrees).

7 had gross sulfonamide crystals in the kidney calyces at slaughter. Cows 151 and 405 had moderate edema at the hilus of the kidney and the kidneys themselves appeared slightly edematous. Tubular dilation of the kidneys was present in cow 971 and microscopic crystals were found in the kidneys of 405. Mild to moderate chronic interstitial nephritis was present in most animals in this group.

Soluble Sulfaquinoxaline (65 mg./kg.).—One cow (cow 973) of 7 had macroscopic crystals in the calyces of the kidneys. Tubular dilation was present in the kidneys of 4 others (cows 844, 339, 147, 148). Crystals were not seen in the kidneys of any of these cattle on microscopic examination.



Graph 5—Distribution pattern for plasma values for cows given daily doses of soluble sulfaquinoxaline by drench. Fifty per cent of the samples assayed 5 mg./100 ml. or higher by day 4.

Soluble Sulfaquinoxaline (30 mg./kg.).—Some tubular dilation or chronic interstitial nephritis, or both, were seen in all cows given this dose. These changes, however, are believed to have been caused by any of a number of pre-experimental causes, since no gross evidence of sulfaquinoxaline crystals was observed.

Soluble Sulfaquinoxaline (15 mg./kg.).—The only change observed in both animals in this group was a chronic interstitial nephritis. Similar degrees of nephritis were seen in certain of the control animals.

Insoluble Sulfaquinoxaline (530 mg./kg.).—Two animals (cows 847, 713) given this dose for six to eight days had macroscopic crystals in the calyces of the kidneys.

Insoluble Sulfaquinoxaline (265 mg./kg.).—The 3 animals (cows 5, 791, 846) medicated at this level had macroscopic crystals in the calyces of the kidneys. Hematuria and erythrocytes were found in the convoluted tubules of cow 5. Microscopic crystals were observed only in the collecting tubules of cow 846.

Insoluble Sulfaquinoxaline (130 mg./kg.).—Of the 2 animals in this group, macroscopic crystals were present in the renal calyces of cow 6. Erythrocytes were observed in the collecting tubules of cow 881.

Insoluble Sulfaquinoxaline (65 mg./kg.).—Cow 7 was given this dosage of drug for eight days. No clinical signs of toxicosis were noticed. Crystals were not found in either kidney, although both kidneys showed typical evidence of chronic pyelonephritis. Microscopic examination of this animal revealed severe, chronic, focal interstitial nephritis. Many collecting tubules contained hyaline casts with atrophied epithelial cells. This infection obviously bore no relation to the experiment.

Ad Libitum Sulfaquinoxaline Water Medication (1 Gm./gal.; 0.025% Solution).—The kidneys of most of the animals in this group were affected with degrees of chronic interstitial nephritis. These changes were no more severe than those seen in control animals.

Sulfonamide Comparison (130 mg./kg.).—Sulfamethazine, sulfathiazole, and sulfa-

bromomethazine were compared with sulfaquinoxaline at the same dosage level (130 mg./kg. daily). Chronic interstitial nephritis was seen in at least 1 animal of each pair, as well as in the sulfaquinoxaline-medicated animal. One animal given sulfamethazine also had erythrocytes in the convoluted tubules. None of the changes observed in these animals was believed indicative of irreversible kidney damage. Pathological changes in the sulfaquinoxaline-medicated animal (cow 405) have been described.

Discussion

It is established that some risk attends the administration of most of the readily absorbable sulfonamides to animals. Reactions occur either in the form of kidney damage or as acute drug shock. The latter is seen more commonly when the drug is given intravenously.

The purpose of this study was to determine the safe oral dose of sulfaquinoxaline for the cow. Early work with sheep by Cameron and McOmie [4] indicated that a single oral dose of sulfaquinoxaline of up to 300 mg. is tolerated well. Subsequent work by Simesen [22] pointed out that oral doses of sulfaquinoxaline of 200 mg. per kilogram (1.5 gr./lb.) in cattle elicited some signs of toxicosis, such as muscular tremor, staggering, and apathy. The work of Stowe *et al.*[25] points out that the toxicity of sulfaquinoxaline seems to be associated particularly with the intravenous use of the drug at doses of 65 to 130 mg. per kilogram. These results showed that crystalluria did occur in their experimental animals and was associated with the period of highest blood concentration. They also found that, in spite of the crystalluria, no hematuria was seen, indicating that the kidneys were not traumatized to the point of causing hemorrhage.

Our data do not support the use of sulfaquinoxaline in doses of 200 mg. per kilogram daily, but show a safe level for clinical use. This is in accord with the accepted use of sulfaquinoxaline, particularly in the treatment of foot rot in cattle. The report of Stowe *et al.*[25] attests to the efficacy of this drug in a dose of 130 mg. per kilogram initially, followed by 65 mg. per kilogram daily thereafter for four to five days.

Conclusions

1) Soluble sulfaquinoxaline, given orally, resulted in no clinically toxic effects in dairy cattle at doses as high as 65 mg. per kilogram for two or three days. Two of 8 animals showed ataxia on the fourth day, and 1 of these had macroscopic crystals in the kidneys.

2) Insoluble sulfaquinoxaline can be given to dairy cattle at doses of 130 mg. per kilogram for as long as six days without causing signs of toxicosis.

3) Cows given any one of three other soluble sulfonamides in doses as high as 130 mg. per kilogram showed no toxicoses.

4) Doses of sulfaquinoxaline as low as 15 mg. per kilogram produce levels in the blood which are detected readily for as long as four to five hours after treatment.

5) There is some indication that sulfaquinoxaline has a tendency to accumulate in the body during the first few days of repeated daily treatments. This seems partly governed by the size of the dose.

6) The inconsistent blood urea nitrogen (BUN) levels may indicate that, despite the presence of gross crystals in the kidney, permanent kidney damage was doubtful and the functional efficiency of this organ was not impaired.

7) Palatability studies have shown that soluble sulfaquinoxaline is acceptable to dairy cattle in concentrations as high as 0.25 Gm. per gallon of drinking water.

8) Sulfaquinoxaline, even in large doses, had no detectable effect on blood clotting time of the cows studied.

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Phenothiazine Treatment of Yearling Beeves on Winter Pastures

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Experiment, Georgia

FREE-CHOICE phenothiazine medication for cattle is a relatively new concept, as compared with the long background of experience in its use to control parasitism in sheep. The literature concerning its use in cattle has been reviewed by Foster.[3]

Phenothiazine has been fed with a grain supplement, in salt, in cottonseed meal and salt, in molasses, and in a mineral mixture. The last method has been found by Herlich and Porter[6] to be preferable to salt. On good quality winter pastures, such as oats, rye grass, or crimson clover, which require no supplementation for good gains, the mineral mixture of salt, bone meal, and limestone is preferred as a vehicle for the phenothiazine.

Low-level phenothiazine administration may result in extra gains and net profits, as reported by Harwood *et al.*[4] However, in experiments conducted by Dennis *et al.*,[2] Herlich and Porter,[6] and Turk *et al.*,[9] no economic advantage resulted from its use.

This study was made to determine the effectiveness of a program of combined free-choice and therapeutic administration of phenothiazine on the control of internal parasites of beef yearlings on winter pastures in Georgia and to determine its effect on average daily gain.

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MATERIALS AND METHODS

Winter grazing tests were conducted for three successive years* to determine the value of phenothiazine treatment for the control of roundworm parasitism in grade Hereford yearlings. In 1954, two types of pastures were used: temporary (mostly oats) and crimson clover; in 1955, rye grass pastures were used, in addition to the temporary and the crimson clover pastures. However, in the latter year, the crimson clover stand was poor, the composition being mostly cheat grass. Therefore, during the 1956 season, fescue pastures were used, instead of crimson clover along with the pastures containing the temporary mixture and those pastures composed of rye grass.

In all three years, the calves were allotted to two groups for each type of pasture on the basis of age, parasite level (as indicated by fecal egg counts), weight, and feeder grade. At the start of the tests, the calves were 10 to 12 months old and weighed 500 to 550 lb.

Each animal in one of the groups on each type of forage was drenched at 28-day intervals with 55 Gm. of phenothiazine in 1954, and with 60 Gm. of the drug in 1955. In 1956, the animals were treated with 60 Gm. of the drug at the beginning of the test and again four months later. In 1954 and 1955, the treated groups also had continuous access to a mineral block containing 7.5 per cent phenothiazine or one part of the anthelmintic to 12 parts of mineral (limestone, bone meal, and salt). In 1956, they had access to a loose mixture of 3 parts salt, 3 parts bone meal, 3 parts limestone, and 1 part phenothiazine. The other group of calves on each pasture was given only loose salt and bone meal in addition to the forage.

To study more fully the effect of phenothiazine on the development of parasitism in the animals, and to determine the potential availability of infective larvae to the cattle, forage samples from each of the pastures were examined periodically, and the larvae present on the forage were recovered and counted. Random collections of forage, cut with a hand clipper in 1954 and with a motorized sickle bar in 1955 and 1956, were processed through a Baermann apparatus to collect nematode larvae. In 1954, six forage samples from different areas in each pasture were clipped at monthly intervals and, in 1955, 24 random grass samples were collected from each pasture, from which six composite samples (0.5 lb.) were examined for larvae. In 1956, the technique was altered,

in that the 24 grass samples from each pasture were collected only from areas on which cattle had apparently grazed within the previous two weeks.

Fecal egg counts were made every 28 days, with large numbers of eggs being counted by the dilution technique and small numbers by the flotation technique of Stoll.[9] The egg counts are shown in the tables as the average egg count for all animals in each pasture for the entire period of the test for that year.

Necropsy studies were made at the end of the grazing season on 2 or 3 animals selected from each lot on the basis of age and weight. The number of different species of worm parasites was estimated by the necropsy technique of Porter.[7] In 1956, a more complete recovery of the immature stages of certain nematodes was obtained by the digestion technique described by Herlich,[8] in conjunction with the freezing procedures of Ciordia *et al.*[1] Since Haemonchus species and the intestinal worms usually were not found in numbers high enough to be of consequence, the tables show the average number of only adult *Ostertagia ostertagi* and *Trichostrongylus axei* and of larvae of the stomach worms.

RESULTS

Phenothiazine Treatment, 1954.—The treated calves on crimson clover consumed an average of 1.03 Gm. of phenothiazine per calf per day, with a range of consumption of 0.69 to 1.33 Gm. during the grazing period, as determined by monthly weighing of the phenothiazine mineral block. On temporary forage, the average daily intake of the drug was 0.44 Gm., with a range of 0.28 to 0.86 Gm. The higher daily consumption of phenothiazine by the calves on crimson clover was reflected in a general reduction of the larval contamination on the pasture and of the parasitic larvae recovered from these calves (table 1). The small amount of the drug consumed by the treated calves on temporary forage had no effect on either the preparasitic or parasitic larval populations.

The therapeutic drenches of phenothiazine apparently were of value in reducing the number of adult worms found in the animals from both pastures; they were especially effective against *T. axei,* but showed erratic action against the medium stomach worm, *O. ostertagi.* Therapeutic medication of the calves on temporary forage was of no value in effecting increased gains, probably because of the low levels of infection in these animals, but an increased gain of 0.2 lb. per calf per day was realized in the treated group on crimson clover.

Phenothiazine Treatment, 1955.—The period of grazing was short in 1955 because of a prolonged drought which delayed seeding of pastures. A small amount of hay was fed through February to those calves on temporary forage.

The calves on crimson clover forage consumed the greatest amount of the drug, 2.09 Gm. per calf per day, with a range of 1.27 to 3.15 Gm. An excellent reduction in the larval contamination on the pasture was observed, although the number of stomach worm larvae recovered at necropsy from the treated animals was almost double

TABLE 1—Effects of Phenothiazine Treatment on Parasitism and Gains of Yearling Beeves on Winter Pastures (1953-54)

Item	Temporary pasture		Crimson clover	
	Treated	Untreated	Treated	Untreated
Test began (date)	12/22/53	12/22/53	1/26/54	1/26/54
No. days on test	146	146	111	111
No. cattle	9	10	7	8
Av. daily gain (lb.)	2.03	2.16	1.80	1.64
Av. daily phenothiazine consumption (Gm./head*)	0.44	.....	1.03	.....
Av. No. roundworm eggs/Gm. of feces†	74	116	58	58
Av. No. larvae recovered from forage (larvae/lb. green forage)	5	5	14	22
No. cattle necropsied	3	3	2	2
Av. No. worms	3,673	6,543	10,954	26,343
Adults	2,273	5,943	5,454	9,943
Larvae	1,400	600	5,500	16,400
Av. No. adult stomach worms**	1,580	5,648	4,551	9,681
Ostertagia ostertagi	1,513	3,923	4,150	2,400
Trichostrongylus axei	67	1,583	400	6,800
Av. No. stomach worm larvae	1,400	600	5,500	16,400

* In addition to amount consumed free choice, each animal received a therapeutic drench containing 55 Gm. of phenothiazine every 28 days; † expressed as the average for entire period of test; ** includes a few Haemonchus spp., but they comprised less than 2 per cent of the total worm load.

TABLE 2—Effects of Phenothiazine Treatment on Parasitism and Gains of Yearling Beeves on Winter Pastures (1955)

Item	Temporary		Rye grass		Crimson clover	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
Test began (date)	2/4/55	2/4/55	3/18/55	3/18/55	3/18/55	3/18/55
No. days on test	98	98	56	56	56	56
No. cattle	11	10	10	10	8	9
Av. daily gain (lb.)	2.11	2.13	3.12	3.29	2.14	2.42
Av. daily phenothiazine consumption (Gm./head*)	0.70	.....	0.54	.....	2.09	.....
Av. No. roundworm eggs/Gm. of feces†	150	203	326	481	164	230
Av. No. larvae recovered from forage (larvae/lb. green forage)	21	119	60	5	11	179
No. cattle necropsied	3	3	3	3	3	3
Av. No. worms	9,892	20,074	19,988	17,046	22,413	30,105
Adults	8,892	16,774	13,855	10,379	11,547	23,505
Larvae	1,000	3,300	6,133	6,667	10,866	6,600
Av. No. adult stomach worms**	4,867	14,700	9,646	7,186	5,007	14,704
Ostertagia ostertagi	4,550	10,033	5,567	5,267	2,800	8,633
Trichostrongylus axei	317	4,667	4,067	1,900	2,200	6,033
Av. No. stomach worm larvae	600	2,300	6,000	5,467	10,733	5,500

* In addition to amount consumed free choice, each animal received a therapeutic drench containing 60 Gm. of phenothiazine every 28 days; † expressed as the average for entire period of test; ** includes a few Haemonchus spp., but they comprised less than 1 per cent of the total worm load.

that found in the untreated animals. The treated animals on temporary forage consumed an average of 0.70 Gm. of the drug, with a range of 0.23 to 1.21 Gm. There was a reduction in larval populations on the pasture and in the animals. The treated calves on rye grass pasture consumed an average of 0.54 Gm. per day, with a range of 0.27 to 0.89 Gm. There was no reduction in parasitism in the treated animals and no effect on larval contamination of the pasture was seen. On temporary and on crimson clover pastures, where the treated animals consumed more of the drug daily, the reduction in the number of adult stomach worms was appreciable, especially with reference to *T. axei*. The phenothiazine medication apparently was of no value in producing increased gains during the limited winter and spring period in which this test was conducted (table 2).

Phenothiazine Treatment, 1956.—Grazing was available for a long winter and spring period in 1956, although some hay had to be fed to the animals in all pastures because of an extended drought during the month of January. Hay feeding was

TABLE 3—Effects of Phenothiazine Treatment on Parasitism and Gains of Yearling Beeves on Winter Pastures (1955-56)

	Temporary		Rye grass-crimson clover		Fescue	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
Test began (date)	12/21/55	12/21/55	1/31/56	1/31/56	12/21/55	12/21/55
No. days on test	152	152	111	111	152	152
No. cattle	9	9	10	9	8	8
Av. daily gain (lb.)	1.98	1.95	2.57	2.74	1.81	1.62
Av. daily phenothiazine consumption (Gm./head*)	1.74	.....	2.79	.....	2.36	.....
Av. No. roundworm eggs/Gm. of feces†	140	205	145	164	165	233
Av. No.larvae recovered from forage (larvae/lb. green forage)	6	4	6	17	12	78
No. cattle necropsied	3	3	3	3	3	3
Av. No. worms	18,611	69,390	14,785	82,978	30,242	194,242
Adults	5,611	12,490	6,685	36,578	10,742	39,775
Larvae	13,000	56,900	8,100	46,400	19,500	154,467
Av. No. adult stomach worms**	5,602	11,618	5,404	36,573	8,408	35,524
Ostertagia ostertagi	4,667	5,567	5,100	18,633	6,800	20,133
Trichostrongylus axei	933	6,033	200	17,933	1,600	15,367
Av. No. stomach worm larvae	13,000	56,767	8,100	46,400	19,233	154,467

* In addition to amount consumed free choice, each animal received a therapeutic drench containing 60 Gm. of phenothiazine at the beginning of the test and again in April; † expressed as the average for the entire period of test; ** includes a few Haemonchus spp., but they comprised less than 1 per cent of the total worm load.

discontinued in the middle of February to the animals on rye grass and temporary pastures, and after the middle of March to those on fescue pastures.

The cattle consumed considerably more phenothiazine in the loose mineral-phenothiazine mixture than had been consumed in the previous years from the phenothiazine-mineral blocks. The treated calves on temporary pasture consumed an average of 1.74 Gm. of the drug per day, with a range of 1.04 to 3.74 Gm. (table 3). No effect on larval contamination of the pasture was seen, probably because comparatively few larvae were recovered from either the treated or untreated pastures. At necropsy, it was found that the treated calves had considerably fewer larvae of the stomach worms and adult *T. axei*, but there was little reduction in the number of *O. ostertagi*. Slightly better gains were made by the treated animals on temporary forage.

The treated calves on the rye grass and crimson clover pastures consumed an average of 2.79 Gm. of the drug daily, with a range of 2.08 to 5.06 Gm., while the treated calves on fescue consumed an average of 2.36 Gm., with a range of 1.52 to 3.39 Gm. On both the fescue and rye grass pastures, the higher levels of drug consumption, combined with the therapeutic treatments, resulted in a reduction in larval contamination on the pastures and a lowering of the number of parasitic larvae and adult stomach worms, especially of *T. axei*. The average daily gain of the treated calves on fescue was significantly greater ($P<0.05$) than that of the untreated group.

In 1956, identification of the larvae recovered from the forage, as well as the parasitic third- and fourth-stage larvae recovered at necropsy, provided an opportunity to study more fully the effect of phenothiazine on the preparasitic and parasitic larval stages. The average daily consumption of 1.74 Gm. of the drug by the animals on temporary forage did not result in over-all control of all the different species present on the pasture, but there was considerable reduction of *T. axei* and *Cooperia puntata* (table 4). The consumption of higher amounts of the drug, 2.36 Gm. and 2.79 Gm. by animals grazing fescue and rye grass, respectively, resulted in good to excellent control of the preparasitic stages on the pastures, and necropsies showed that the combined therapeutic and low-level administration of the drug had considerably reduced the number of parasitic stomach worm larvae.

The number of adult and larval intestinal worms found in the animals in 1956 was never over 8 per cent of the total worm load, and the number of Cooperia spp. larvae recovered was insignificant (table 4). Yet, from 48 to 69 per cent of the larvae recovered from the pastures, treated and untreated, were *C. punctata* and *Cooperia oncophora*. Presumably, the animals acquired an immunity against Cooperia spp. that was sufficient to remove any infections with these species and to prevent subsequent reinfections.

Effect of Pasture on Parasitism.—In a comparison of the average number of worms recovered from the untreated animals for each of the different years (tables 1, 2, 3), the results generally confirmed previous investigations.[10] In 1954 and 1955, the calves on crimson clover had more worms than the calves on temporary or rye grass forages. Also, in the former year, the calves on crimson clover made lower

TABLE 4—Relationship Between Numbers of Infective Larvae Recovered from Green Forage and Numbers of Parasitic Larvae Recovered at Necropsy (1956)

	Temporary		Rye grass-crimson clover		Fescue	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
Total No. larvae/lb. green forage*	54	38	43	117	106	602
Osteragia ostertagi	18	3	5	34	42	198
Trichostrongylus axei	1	9	13	27	11	106
Cooperia oncophora	34	20	5	8	52	240
Cooperia punctata	1	6	20	48	1	58
Av. No. parasitic larvae recovered at necropsy†	13,000	56,900	8,100	46,400	19,500	154,467
Osteragia ostertagi	10,920	53,361	7,938	40,368	16,925	132,842
Trichostrongylus axei	650	1,135	0	1,856	385	7,723
Unknown (from stomach)	1,430	2,271	162	4,176	1,923	13,902
Cooperia spp.	0	133	0	0	267	0

* Total number of larvae recovered per pound of forage during entire period of test; † fourth-stage larvae were predominantly present, only 1 to 19 per cent of the *O. ostertagi* larvae being in third stage.

average daily gains than similar calves on temporary forage but, during the short period of the experiment the following year, no relationship between weight gain and parasitism was found. In 1956, the untreated animals on fescue made the least gain and had more worms at necropsy than their counterparts on temporary and rye grass-crimson clover forages.

Statistical Analyses.—In a determination of the significance, if any, in relation to particular species of adult stomach worms and larvae of the stomach worms, the three years' results were combined to give adequate numbers to partially offset any natural variation encountered. No statistical significance was found between the number of adult and larval intestinal worms recovered from treated and untreated animals or between the numbers found in untreated animals on the different pastures. However, the number of stomach worm larvae recovered was significantly lower ($P<0.05$) in the treated animals (tables 1, 2, 3), and the lower numbers of *O. ostertagi* and *T. axei* recovered from treated animals was highly significant ($P<0.01$). The small stomach worm, *T. axei*, was so satisfactorily controlled by the drug that the differences between treated and untreated animals on temporary pastures and between treated and untreated animals on crimson clover were highly significant ($P<0.01$). The only test in which treated animals made average daily weight gains statistically greater than those made by untreated animals occurred on fescue pastures (table 3).

Considering the differences in average daily gain between the untreated animals on each of the different types of pastures, it was found that the calves on rye grass made significantly greater gains ($P<0.01$) than the calves grazing temporary, crimson clover, or fescue pastures (tables 2, 3). Also, the calves grazing the temporary pasture made average daily gains significantly greater ($P<0.01$) than those made by the animals on the fescue pastures.

With reference to stomach worm larvae and adult *T. axei*, the calves on temporary forage had significantly fewer worms ($P<0.01$) than the calves grazing fescue grass (table 3). In the same two groups, *O. ostertagi* was also found in significantly ($P<0.02$) fewer numbers from the calves on temporary forage. The calves grazing rye grass had significantly ($P<0.05$) fewer total worm numbers and significantly ($P<0.02$) fewer stomach worm larvae than the calves grazing on fescue.

The statistical results generally show that the type of pasture grazed was more important in increasing average daily gain than was the phenothiazine treatment, although the combined therapeutic and low-level administration of phenothiazine was as effective in the control of adult stomach worms as the type of pasture grazed by the animals. However, the type of pasture had more influence than the anthelmintic treatment with regard to the number of larval stomach worms recovered at necropsy. The nutritional effect of the various forages on the development of the infective larvae after ingestion by the host requires intensive investigation.

DISCUSSION

The greatest reduction in larval contamination on pasture was 94 per cent. This apparently was the result of the average daily consumption of 2.09 Gm. of phenothiazine by the treated animals on crimson clover in 1955 (table 2). The data indicate that this or a higher level of phenothiazine intake is necessary for the satisfactory control of the preparasitic stages on the pasture (tables 2, 3).

The best control of the parasitic larvae of the stomach worms and adult *O. ostertagi* and *T. axei* occurred in 1956 under the regimen of free-choice phenothiazine in a loose mixture of minerals in addition to two therapeutic treatments, one at the beginning of the test and another four months later (table 3). An 88 per cent reduction in parasitic larvae of the stomach worms was evident in the treated animals on fescue, where consumption of the drug averaged 2.36 Gm. per day. *Ostertagia ostertagi* and *T. axei* were reduced by 73 per cent and 99 per cent, respectively, in the treated animals grazing rye grass-crimson clover, where the drug was consumed at an average rate of 2.79 Gm. per day.

The relationship of the type of pasture grazed to the number of parasitic larvae of the stomach worms recovered at necropsy generally was evident in all years (tables 1, 2, 3). The untreated animals on temporary and rye grass forages had fewer stomach worm larvae than similar animals on crimson clover or fescue pastures. This was especially evident in 1954 (table 1),

when the untreated calves on temporary forage had 91 per cent fewer stomach worm larvae than the untreated animals on crimson clover. Also, in 1956 (table 3), the untreated calves on rye grass and on temporary pastures had 70 per cent and 63 per cent, respectively, fewer larvae of the stomach worms than their counterparts on fescue forage.

On the basis of these experiments, the use of the phenothiazine mineral blocks can not be recommended over the loose mixture of the drug and mineral. Although the anthelmintic was convenient to use in the mineral block form, the low daily consumption did not give the desired results.

CONCLUSIONS

The low-level feeding of phenothiazine to beef yearlings on winter pastures was not of economic benefit, except to those animals grazing on fescue forage. The nutritional adequacy of the temporary forages (oats and rye grass) and crimson clover appeared to influence cattle gains more than the anthelmintic or larvicidal action of the drug. However, the parasitism of cattle grazing on these forages was at a subclinical level, so that any possible benefit from the drug could not be ascertained.

The combined low-level and therapeutic administration of the drug was found to be effective in larvicidal action on the preinfective larval stages on pasture and anthelmintic action on the adult stomach worms, if the average daily intake per animal was 2 Gm. or more, and therapeutic drenching was conducted at least twice during the winter and spring grazing period. Although the number of parasitic larvae of the stomach worms was considerably reduced by the drug, especially when the average daily intake was higher than 2 Gm., the type of pasture grazed by the animals appeared to be more closely correlated with the number of parasitic larvae of the stomach worms which were found at necropsy.

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Correction—Development of the Heart in Bovine Embryos

In the article, "Observations on the Early Development of the Heart in Bovine Embryos with Six to Twenty Paired Somites," by Marlene R. Grimes, J. S. Greenstein, and R. C. Foley, in the July, 1958, issue of the *American Journal of Veterinary Research* (p. 591), the second paragraph of the Summary, beginning with the sentence starting on line 13, should read "*As the atria fuse and come to lie dorsal to this loop, the heart assumes a spiraled S shape. By the 20-somite stage, the sinus venosus is distinguishable, and the heart is capable of maintaining some circulation of blood throughout the yolk sac, central nervous system, liver primordium, and allantoic diverticulum.*"

The Effect of Experimental Infection with *Ostertagia Ostertagi* and *Trichostrongylus Axei* in Calves on Weight Gain, Feed Consumption, and Feed Utilization

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THE ECONOMIC LOSS caused by clinical parasitism has long been recognized, but losses from varying degrees of subclinical parasitism are not so easily recognized and evaluated. Andrews and Maldonado [2] compared calves with light and heavy infections of *Oesophagostomum radiatum* and found that calves with light infections gained more than those with heavy infections, but not as much as the controls. Spedding [7] reported that subclinical infections of lambs reduced weight gain.

Ostertagia ostertagi and *Trichostrongylus axei* occur in the abomasa of cattle and are the predominating species found in cattle of the Gulf Coastal Plains area of Texas. Andrews *et al.*[4] reported an average of 76,612 *O. osteragi* and 41,360 *T. axei* in 14 Georgia cattle. These two species accounted for 63 per cent of the six species of gastrointestinal parasites found.

Subclinical parasitism with the gastrointestinal nematode parasites is general in the Gulf Coastal Plains area of Texas and, since *O. ostertagi* and *T. axei* are found in greater numbers, a study of the effects of subclinical parasitism caused by these two species appeared desirable.

MATERIALS AND METHODS

Day-old Jersey bull calves were obtained and raised in individual pens on concrete floors under conditions that excluded infection with the nematode parasites, with the possible exception of Strongyloides organisms.

Weighing of the calves on two consecutive days each week was begun at 3 months of age, and the amount of feed given daily was adjusted to the weight of each calf. Approximately two months before the beginning of each test (3 months before the beginning of the experimental infection) the ration was standardized for that experiment.

In experiments 1 (5 calves) and 2 (8 calves), the ration included rolled oats, cottonseed meal, ground milo, dehydrated alfalfa, and minerals (bone meal and salt), and contained approximately 11 per cent digestible protein (DP) and 65 per cent total digestible nutrients (TDN). In experiment 3 (16 calves), the ration was composed of equal parts of ground milo and alfalfa plus bone meal and salt, and also contained approximately 11 per cent DP and 65 per cent TDN. In experiment 4, two groups of 8 calves each were used. One group was given ground milo, alfalfa, and mineral ration (11% DP and 65% TDN). The second group was fed a ration of 20 per cent cottonseed meal, 30 per cent cottonseed hulls, 50 per cent ground milo, and mineral, containing approximately 7 per cent DP and 65 per cent TDN.

During the month before the test began, an attempt was made to place the calves in equivalent groups (for each experiment) on the basis of rate of gain and feed utilization.

The experimental infection of the calves was carried out by the established procedures of determining the number of larvae in the inoculum and feeding it with a metal pipette. The ratio of Ostertagia and Trichostrongylus larvae fed was one to two. In calves given both, the number was reduced to one half for each species.

In the first three experiments, *T. axei* larvae were given to one group of calves, *O. ostertagi* to another, and larvae of both genera to a third group. Calves of experiment 4 were given only *T. axei* larvae.

The test period was divided into a four-week preinfection, a three-week prepatent, and a five-week patent period. A definite and accurate prepatent period could not be designated, due to the prolonged period of larvae administration in some cases. Records were kept of the weekly weights of calves, daily feed consumption, and biweekly worm egg-per-gram fecal counts. At the end of all experiments except experiment 4, the calves were slaughtered and worm burden counts were made of all infected calves.

RESULTS

The results are shown (table 1). With the possible exception of 1 infected calf in experiment 1, the degree of parasitism pro-

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TABLE 1—Data on Calves Experimentally Infected with Ostertagia Ostertagi and Trichostrongylus Axei

No. calves	Age at infection	Av. weight	4-wk. preinfection period: Av. daily gain	Daily feed intake	Feed util.	3-wk. prepatent period: Av. daily gain	Daily feed intake	Feed util.	5-wk. patent period: Av. daily gain	Daily feed intake	Feed util.	Period of larvae administration	No. larvae given: Ostertagia	Trichostrongylus	Eggs per gram: Max.	Av.	No. worms recovered	No. days infected
Experiment 1—11 Per Cent DP and 65 Per Cent TDN.																		
2	197	169	1.18	6.30	0.19	1.33	6.49	0.19	1.33	7.03	0.19		.....	.....	.....	.....	.....	.....
1	198	194	1.11	7.21	0.15	1.50	7.38	0.20	1.37	8.04	0.17	Nov. 29, 1954,	118,000	.....	267	99	180	100
1	189	142	1.10	5.44	0.20	2.00	5.78	0.34	0.11	6.08	0.02	to	.....	242,000	114	31	2,460	100
1	191	165	1.04	6.21	0.16	1.47	6.36	0.23	1.03	6.92	0.15	Dec. 24, 1954	61,000	121,800	151	64	6,010	100
Experiment 2—11 Per Cent DP and 65 Per Cent TDN.																		
2	246	230	1.35	6.87	0.19	1.09	7.44	0.14	1.63	8.90	0.18		.....	.....	.....	.....	.....	.....
2	249	233	1.62	6.96	0.23	1.33	8.12	0.15	1.44	8.94	0.16	Jan. 17, 1955,	173,000	.....	342	172	4,270	63
2	250	246	1.57	7.30	0.21	1.47	8.50	0.17	1.50	9.49	0.15	to	.....	360,000	45	18	4,470	60
2	248	221	1.30	6.61	0.19	1.01	7.48	0.18	1.44	8.46	0.17	Feb. 11, 1955	86,700	180,000	557	137	11,020	63
Experiment 3—11 Per Cent DP and 65 Per Cent TDN.																		
4	269	281	1.85	7.36	0.25	1.59	10.45	0.15	1.63	12.06	0.13		.....	.....	.....	.....	.....	.....
4	268	278	1.63	7.70	0.22	1.63	10.06	0.16	1.42	10.76	0.13	June 20, 1955,	224,100	.....	308	146	23,070	63
4	276	280	1.89	7.34	0.25	1.81	10.27	0.18	1.72	11.30	0.14	to	.....	448,400	110	50	28,780	63
4	276	282	1.91	7.20	0.26	1.70	10.29	0.16	1.62	11.50	0.14	July 22, 1955	110,300	223,200	198	102	35,000	63
Experiment 4—11 Per Cent DP and 65 Per Cent TDN.																		
4	270	284	2.17	9.21	0.24	1.71	10.53	0.16	2.32	12.15	0.17		.....	.....	.....	.....	.....	.....
4	269	297	1.68	9.53	0.17	0.20	8.11	0.02	−0.76	5.67	−0.20	June,* 1956	.....	1,000,000	1,175	445	.....	.....
Seven Per Cent DP and 65 Per Cent TDN.																		
4	274	274	1.19	9.19	0.12	1.02	9.82	0.10	1.34	10.78	0.12		.....	.....	.....	.....	.....	.....
4	266	253	1.04	9.11	0.11	0.68	9.06	0.07	−0.46	6.40	−0.25	June,* 1956	.....	1,000,000	5,675	1,144	.....	.....

* One calf of each higher and lower protein group was given 1 million larvae on June 11 to 13, 1 of each group was given the larvae on June 14 to 15, 1 of each was given larvae on June 17 to 18, and 2 were given larvae on June 20.

duced in calves of experiments 1, 2, and 3 did not appear to affect the rates of gain, feed intake, and feed utilization.

In experiment 4, during the four-week preinfection period, feed intake was approximately the same in calves given the higher and lower protein rations; the rate of gain was 1.68 to 1.04 lb. daily, and feed utilization was 0.17 to 0.11 lb. per pound of feed. During the prepatent period, comparisons of the control and infected calves given the higher protein ration showed the daily rates of gain to be 1.71 and 0.20 lb., the feed intake to be 10.53 and 8.11 lb., and the feed utilization to be 0.16 and 0.02 lb., respectively. For control and infected calves given the lower protein ration, the comparisons showed the rates of gain to be 1.02 and 0.68 lb., the feed intake to be 9.82 and 9.06 lb., and the feed utilization to be 0.10 and 0.08 lb., respectively.

During the patent period, comparisons of control and infected calves given the higher protein ration showed the rates of gain to be 2.32 and —0.76 lb., the feed intake to be 12.15 and 5.67 lb., and the feed utilization to be 0.17 and —0.20 lb., respectively. Comparisons of control and infected calves given the lower protein ration showed the rates of gain to be 1.34 and —0.46 lb., the feed intake to be 10.78 and 6.40 lb., and the feed utilization to be 0.12 and —0.25 lb., respectively.

The maximum and average worm egg-per-gram counts of fecal samples of infected calves given the higher protein ration were 1,175 and 445, respectively. Those of calves given the lower protein ration were 5,675 and 1,144, respectively.

Signs of clinical parasitism, such as scouring and poor condition, occurred in 2 of the 4 infected calves given the lower protein ration, and in only 1 calf of the group given the higher protein ration.

Discussion

Threlkeld and Johnson [8] experimentally infected 5 calves, 2 to 6 months old, with 12,408, 49,390, 129,400, 216,373, and 14,800 *O. ostertagi* larvae, respectively, without producing clinical parasitism.

Doran [5] experimentally infected 10 calves, 3 to 6 months old, with *T. axei* larvae and reported that infection with less than 750,000 larvae did not produce clinical signs, but did produce gross pathological changes in the abomasa. Clinical signs occurred when 750,000 larvae were given, and death resulted when 1.0 and 1.5 million larvae were given.

Other than the scouring and loss of condition in 3 of the calves given 1 million *T. axei* larvae, signs of parasitism were slight in the other infected calves, as indicated by the failures of three persons (one veterinarian and two experienced cattlemen) to differentiate between some of the infected and noninfected calves. The average percentage of accuracy of the three persons judging was 66 per cent in calves given the lower protein ration and 83 per cent in the calves given the higher protein ration. Although there was greater loss of weight and reduced feed utilization in the infected calves given the higher protein ration, this was primarily due to the loss of the extra fat and bloom which these calves had, and would not have been noticeable had not the controls been available for comparison. It was more difficult to differentiate between the unthrifty condition of infected and noninfected calves given the lower protein ration.

Subclinical parasitism, of a degree indicated by the average number of worms recovered at necropsy (28,780) of the infected calves of experiment 3, seemed to be of economic importance, but the way in which loss of weight or harm to the host is brought about is not known. Gibson [6] considers the depression of appetite an important factor in *T. axei* infections in sheep. If natural infection of calves on pasture reduces the effort involved in grazing, thereby lowering the feed intake with its subsequent effects, then the absence of signs of parasitism in the calves of experiment 3 may be due to the fact that little effort and energy were involved in obtaining the feed and water.

Andrews [1] and Andrews *et al.*[3], using lambs infected with *Cooperia certicei* and *Trichostrongylus colubriformis*, reported that feed utilization was reduced in infected lambs. Feed utilization also was reduced in the infected calves of experiment 4. However, it appeared that the infected calves given the lower protein ration were more efficient in feed utilization than those given the higher protein ration.

Summary

Experimental infection of 9-month-old Jersey calves, on a good ration, with 224,000 *Ostertagia ostertagi* larvae, 448,000 *Trichostrongylus axei* larvae, or 111,000

O. ostertagi and 224,000 *T. axei* larvae resulted in no change in average daily weight gain, feed intake, and feed utilization during an eight-week postinfection period.

Of the 9-month-old calves fed a ration of 11 per cent digestible protein (DP) and 65 per cent total digestible nutrients (TDN) and given 1 million *T. axei* larvae, 1 showed clinical parasitism and 3 showed only subclinical parasitism. Infected calves lost 0.4 lb., and the average daily feed intake was reduced 43 per cent. Feed utilization was 0.16 lb. per pound of feed in noninfected and −0.06 lb. in infected calves.

Of the 9-month-old calves fed a ration of 7 per cent DP and 65 per cent TDN and given 1 million *T. axei* larvae, 2 showed clinical and 2 showed subclinical parasitism. The average daily gain in the noninfected calves was 1.22 lb.; infected calves lost an average of 0.03 lb., and feed intake was reduced 29 per cent. Feed utilization was 0.10 lb. per pound of feed in noninfected and −0.07 lb. in infected calves.

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Studies on Shipping Fever of Cattle. I. The Experimental Exposure of Cattle with Various Cultures of Pasteurella

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THE VIRULENCE of Pasteurella organisms has been shown to vary widely.[9,12,14] Pasteurella organisms have been recovered from animals with shipping fever.[6,10,14,15] Carter[6] observed that, in 26 cattle with typical cases of shipping fever, *Pasteurella hemolytica* was isolated from 15 and *Pasteurella multocida* from 10. Carter and McSherry[10] recovered *Past. hemolytica* from the nasal swabs of 27 of 33 cattle with shipping fever. King *et al.* isolated Pasteurella organisms from the lung tissues of 8 of 9 animals dead from shipping fever.[14]

The role of these organisms in this disease is not clear. Whether they are merely secondary invaders or, under certain conditions of stress, are able to invade their host and become pathogenic, is not known. The purpose of this work was to add to the information concerning the pathogenic potential of Pasteurella organisms in the production of shipping fever.

MATERIALS AND METHODS

Cultures 49M (*Past. multocida*) and 49H (*Past. hemolytica*) were isolated from a calf experimentally injected with material from an animal with an acute case of shipping fever. Culture 113M (*Past. multocida*) was isolated from a feeder calf which died of shipping fever in Ohio. The following cultures were obtained from Carter:* culture 93 (*Past. multocida*, type D, bovine), isolated from an animal with shipping fever in Iowa; culture 8455 (*Past. hemolytica*), isolated from an animal with shipping fever in Ontario; and culture 1578 (*Past. multocida*, type A), isolated from a cow with pneumonia in Ontario.

The cultures were grown and maintained on blood agar plates and in brain-heart infusion broth (Difco) enriched with 10 per cent turkey or rabbit serum. During this study, cultures were transferred and checked a minimum of once each week. The cultural and physiological characteristics of these strains agreed with those described for *Past. multocida* and *Past. hemolytica* in "Bergey's Manual."[5] All cultures were incubated at 37 C.

Inocula for injection of experimental animals were prepared in brain-heart infusion broth or in a phosphate buffer solution where animal tissues were used. Cultures were passed periodically through mice and rabbits. Materials used for inoculation were incubated for 18 to 24 hours at 37 C. A MacFarland nephelometer reading indicated that the washed suspension contained between 12 to 18 billion organisms per milliliter. When animal tissue was used for inoculum, it was macerated in TenBroeck tissue grinders, and a phosphate buffer diluent was added to make a 10 per cent suspension. Of the several different routes of inoculation used, more than one route in most animals, the intratracheal and subcutaneous routes were used most frequently.

In some calves, cortisone acetate was used as a stressing agent at the rate of 1 mg. per pound of body weight every 24 hours for three days. Stilbestrol (75 mg.) was given to 1 calf for three days at 24-hour intervals. The last injection of these agents was given on the same day that the inoculum was given.

Sixteen healthy dairy calves, 4 to 11 months old, were used. They were raised at the Ohio Agricultural Experiment Station. Most of them were killed for necropsy after being observed for 30 days after inoculations; a few were sent to slaughter after three weeks.

Female Swiss white mice, 4 to 5 weeks old, were used for titration of virulence of the various cultures. They were injected with 0.2 ml. of the various dilutions for titration studies.

The rabbits were mature domestic albinos from a colony free of Pasteurella infections.

RESULTS AND OBSERVATIONS

In the first trial, 2 calves were injected with cultures 49M (*Past. multocida*) and 49H (*Past. hemolytica*), grown on brain-heart infusion broth. Each calf was given 2 cc. of an 18-hour culture of brain-heart infusion broth subcutaneously and 10 cc. intratracheally. These animals were observed and temperatures taken for three to four weeks; no visible reaction or febrile response was observed. Blood samples were taken from the jugular veins on the second

From the Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster.

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TABLE 1—Summary of Experimental Inoculations of Calves with Pasteurella Multocida and Pasteurella Hemolytica

Calf (No.)	Inoculum	Culture	Stressing agent	Route of inoculation
1	Broth culture	M	.....	s.c., i.t.
2	Broth culture	H	.....	s.c., i.t.
3	Broth culture + RL	M	.....	s.c., i.m., i.t.
4	Broth culture	H	.....	s.c., i.m., i.t.
5	Broth culture	M	.....	s.c., i.t., i.p.
6	Broth culture	M	.....	s.c., i.t.
7	Broth culture	M	.....	s.c., i.t.
8	Broth culture + ML	M	.....	s.c., i.t.
9	Broth culture + BL	M, H	.....	s.c., i.t.
10	Broth culture + ML	M	.....	s.c., i.t.
11	Broth culture	M	Cortisone	s.c., i.t.
12	Broth culture	M	Cortisone	s.c., i.t.
13	Broth culture	M	Cortisone	s.c., i.t.
14	Broth culture	M	Stilbestrol	s.c., i.t.
15	Broth culture	H	Cortisone	i.t.
16	Broth culture	M	Cortisone	i.t.

M = *Pasteurella multocida*; H = *Pasteurella hemolytica*; RL = rabbit liver suspension; ML = mouse liver suspension; BL = bovine lung suspension; s.c. = subcutaneous; i.t. = intratracheal; i.p. = intraperitoneal; i.m. = intramuscular.

and sixth days, with no recovery of the challenging organisms.

In a second attempt to establish infection, a similar procedure was followed, except that each animal was given an intramuscular injection and culture 49M was given intratracheally as a rabbit liver suspension. The liver suspension was obtained from a rabbit which died 18 hours after injection of culture 49M. No reaction was observed; blood cultures again were negative for Pasteurella organisms.

Three additional calves were inoculated with culture 93; 1 was inoculated intraperitoneally and intratracheally, another subcutaneously and intratracheally, and the third intratracheally. None showed signs of disease. Another trial was made, using both broth culture and liver suspension of mice dead or acutely ill from earlier injections of this organism.

Culture 113M was obtained from a feeder calf with acute shipping fever, both before and after death. A suspension of lung tissue from this calf and 10 ml. of broth culture were injected subcutaneously and intratracheally into 1 calf. Another calf was injected with broth culture and lung tissue from mice dead or dying from this infection. No reaction was noticed in either calf.

Since hormones have been shown to act as stress factors,[13] 3 calves were injected with cortisone and 1 with diethylstilbestrol. Two of the cortisone-treated calves were given broth cultures of culture 93, subcutaneously and intratracheally, simultaneously with the last injection of cortisone. No reaction was noticed. The other calves treated with cortisone and diethylstilbestrol were given culture 113M similarly and showed no reactions. Two additional calves were given cortisone and then injected intratracheally with cultures 8455 and 1578, with no reactions. The experiments are summarized (table 1). All cultures were titrated for virulence and the results are summarized (table 2).

TABLE 2—The Virulence of Different Pasteurella Organisms for Mice

Dilution	*P. multocida* (49M)	*P. hemolytica* (49H)	*P. multocida* (113)	*P. multocida* (93)	*P. hemolytica* (8455)	*P. multocida* (1578)
10^{-1}	0/5*	4/5	0/5	0/5	5/5	0/5
10^{-2}	0/5	4/5	0/5	0/5	5/5	0/5
10^{-3}	0/5	5/5	0/5	0/5	0/5	1/5
10^{-4}	0/5	4/5	0/5	0/5	0/5	0/5
10^{-5}	0/5	3/5	0/5	1/5	5/5	1/5
10^{-6}	0/5	4/5	0/5	1/5	4/5	0/5
10^{-7}	0/5	4/5	0/5	0/5	3/5	1/5
10^{-8}	1/5	5/5	1/5	1/5	3/5	3/5
10^{-9}	3/5	5/5	2/5	4/5	5/5	5/5
10^{-10}	5/5	5/5	5/5	5/5	5/5	5/5

* Numerator = number of survivors, denominator = the number of animals inoculated (all mice were observed for a period of 7 days).

All calves were either sent to slaughter or necropsied. No visible lesions of pneumonia were found in any of these animals. Bacteriological examination of the lungs 21 to 30 days after exposure failed to reveal the presence of Pasteurella organisms in the lungs of these calves. No significant changes were noticed on periodic blood examinations of total leukocyte and differential examinations.[4]

Discussion

No signs of disease were produced in 16 calves inoculated by various routes with and without hormone stress. It is apparent from these trials that the Pasteurella organisms did not produce respiratory signs or lesions in exposed animals. It would appear therefore, that these organisms could not be primary agents in shipping fever. The cultures of *Past. multocida* showed varying degrees of virulence for mice; culture 113 was especially virulent. Cultures of *Past. hemolytica* were found to be of low virulence for mice. These findings are in accord with those reported previously.[15]

While it is possible that the growth in artificial mediums could have resulted in loss of virulence, it would be difficult to explain why culture 113 (used as a lung suspension) would cause no disease, since it was isolated in pure culture from the lungs of a calf with a naturally occurring case.

Culture 1578 was used to check virulence of organisms belonging to the type A group. Type D was represented by culture 93. Carter [8] has reported that types A and D were predominant in North American cattle. The results of typing strains of *Past. hemolytica* by Carter [7] indicate that one serological type is representative of all strains examined. In this study, the *Past. hemolytica* used was obtained locally; culture 8455 was obtained from Carter. No signs of disease were evident as the result of inoculation with any of these organisms.

Carter was able to produce clinical infections in 8 of 19 calves exposed to *Past. hemolytica*. Bacteriological and pathological observations on these calves suggested that some experimental calves had been affected with a chronic pneumonia prior to exposure. He postulated that this chronic pneumonia contributed to *Past. hemolytica* infections. Under the conditions of this experiment, no pneumonic lesions were found in any of the calves at the time of slaughter or necropsy. That Pasteurella organisms are primary inciting disease agents in North American cattle is questionable.[1-3] What role they may play as pathogens and in individual host reaction requires more research.

Summary

Sixteen calves were exposed to cultures of *Pasteurella hemolytica* and *Pasteurella multocida* without producing illness. Cortisone and diethylstilbestrol, used as stressing agents, did not seem to alter the resistance of animals to the challenge. At necropsy, no indication of pneumonia was found. No significant temperature reaction was noticed and the blood picture was not altered to any extent. Virulence titrations in mice, for the cultures used, showed that *Past. multocida* strains varied in virulence, while *Past. hemolytica* was found relatively nonpathogenic.

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Studies on Shipping Fever of Cattle. II. Isolation of Pleuropneumonia-like Organisms

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PLEUROPNEUMONIA-LIKE ORGANISMS (PPLO) are associated with respiratory diseases of various animals and birds. Bridre and Donatien [1] reported, in 1923, that the causal agent of contagious agalactia was similar to that of bovine pleuropneumonia. Edward [5] pointed out that the disease in goats is caused by bovine organisms that have been adapted to goats. The first report of the isolation of PPLO from the respiratory tracts of sheep was made in Canada.[7] Pleuropneumonia-like organisms were recovered from the joints, kidneys, and spleen of a calf with arthritis and bronchopneumonia.[13] Carter and McSherry [2] isolated PPLO from nasal swabs of cattle with shipping fever. The actual role of PPLO in this condition is not understood clearly.

The purpose of this paper is to report the isolation of PPLO from animals with shipping fever and some information on the pathogenicity of these organisms.

MATERIALS AND METHODS

Pleuropneumonia-like organisms were obtained either with sterile cotton swabs, moistened with buffered saline solution, which were inserted well into the nostrils of 7 steers with shipping fever, or with pieces of lung tissues taken from these steers at necropsy. These specimens were introduced into tubes containing 8 to 10 ml. of Morton's[12] modified broth[10] containing thallium acetate at a dilution of 1:2,000, penicillin at a concentration of 200 units per milliliter, and 2 per cent Difco serum fraction. The inoculated tubes were incubated at 37 C. for as long as seven days. However, after two, three, and seven days of incubation, a loopful from each of the inoculated tubes was streaked on PPLO agar plates enriched with 2 per cent serum fraction. After another 48 hours' incubation, the plates were examined microscopically under low power (x 100) for PPLO colonies. If PPLO were not observed after seven days of incubation, the plate was discarded as negative for PPLO. In addition to colony growth, biochemical reactions were studied, using 14 different sugars in phenol red broth [8] containing 1.5 per cent serum fraction and having a pH range of 7.2 to 7.3.

The properties and the range of specificity of bovine PPLO were determined in calves, developing chicken embryos, hamsters, guinea pigs, turkeys, rabbits, and mice. The ability of PPLO to pass through various sterilizing filters was determined, using Seitz pad and Selas No. 01, 015, 02, and 03 filters.

Three calves, 4 to 8 months old, used for artificial exposure, were maintained in separate pens in an isolation building and were fed a ration of mixed hay and a 15 per cent grain mixture without antibiotics. They apparently were healthy, with no history of shipping fever.

Calf 1 was inoculated intratracheally with 4 ml. and intranasally with 6 ml. of a combination of the three recovered bovine strains of PPLO in phenol red maltose broth. Calf 2 was inoculated similarly with half the dosage of PPLO strains given to calf 1, plus similar quantities of *Pasteurella multocida*, in brain-heart infusion broth. Calf 3 was inoculated exactly as was calf 2 and, in addition, was given 0.5 Gm. of cortisone acetate intramuscularly as a stressing agent, the day before and again at the time of exposure to the cultures.

The fertile eggs were obtained from chickens fed antibiotic-free ration and which had no signs of chronic respiratory disease (CRD).

RESULTS

Three bovine strains of PPLO, isolated from 7 steers with shipping fever, developed characteristic PPLO colonies. A dense central zone and a lighter peripheral zone appeared on serum agar plates after 48 to 72 hours' incubation (fig. 1).

These strains of PPLO did not produce gas in any of the sugars tested. However, all three strains produced acid in glucose, maltose, sucrose, and trehalose (table 1). Acidity was indicated by the color change of the phenol red indicator, as well as by the pH measurements, two or three days after inoculation. Acidity was pronounced in these four fermented sugars, with a pH range of 5.8 to 6.1, while the pH of the control tube was 7.2. Pleuropneumonia-like organism broth culture had the ability to

From the Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster.

pass through the Seitz pad and Selas No. 01, 015, and 02 filters, but were retained by Selas No. 03 filters at a negative pressure of 50 to 60 mm. of mercury. The filterability of these organisms was determined by the recovery of PPLO from the filtrate and the ability to produce acid in phenol red maltose medium.

Bovine PPLO were propagated successfully in 7-day-old chicken embryos. In the first egg yolk passage, 2 of 12 inoculated embryos died seven and eight days after inoculation. As the serial passage progressed, the mortality increased. At the sixth passage, all embryos were dying four and five days after inoculation.

The allantoic membrane was cloudy and thickened, and the yolk material was thin and watery. Dwarfing of the embryos and subcutaneous ecchymoses were observed frequently; the liver and kidneys were congested and dark. Pleuropneumonia-like organisms were recovered from the allantoic fluid, yolk material, and the embryo. The 50 per cent end point of the eighth passage was measured *in ovo* and found to be $10^{-3.8}$.

The range of pathogenicity of the bovine strains of PPLO was determined in various laboratory animals. Three-week-old mice were infected by intranasal instillation only when strain 53 was used. On necropsy, one week after exposure, small hemorrhagic spots were observed in the lungs and PPLO were recovered from their lung tissues. Hamsters, rabbits, guinea pigs, turkey poults, and old mice seemed to be refractory to all the strains. Pleuropneumonia-like organisms were not recovered from their lung tissues. However, antibodies against PPLO were demonstrated in 10 of 24 turkeys, using the hemagglutination-inhibition test,[6] at a titer of 1:48 to 1:160 serum dilution. These birds did not show any apparent clinical signs of turkey sinusitis, but antibodies against PPLO were demonstrated four weeks after intrasinus inoculation.

At necropsy, three weeks after inoculation, pneumonia was not observed in any of the 3 exposed calves. Hematological studies of calf 1 revealed no abnormalities, and the lungs were essentially normal. Pleuropneumonia-like organisms were not recovered from the lung tissues.

Calf 2 showed signs of anorexia on the third and fourth days and its temperature ranged from 104.0 to 104.4 F., but pulmo-

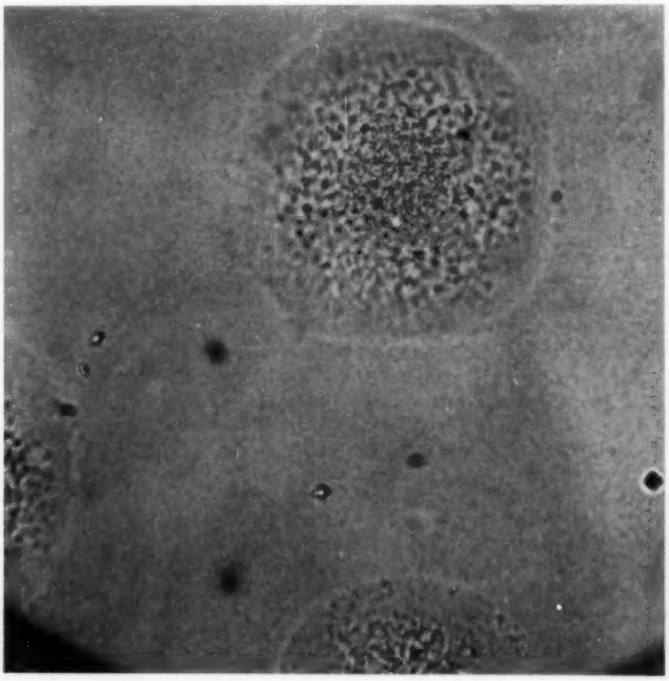

Fig. 1—Colonies of bovine pleuropneumonia-like organisms (strain 53) on agar plate after three days of incubation. Transmitted light; x 350.

TABLE 1—Ability of Three Bovine Strains of PPLO to Ferment Carbohydrates on Incubation for 72 Hours and to Pass Through Filters

Item	PPLO strains 53	58	59
Carbohydrate			
Arabinose	−	−	−
Galactose	−	−	−
Glucose	+	+	+
Lactose	−	−	−
Levulose	−	−	−
Inositol	−	−	−
Maltose	+	+	+
Mannose	−	−	−
Salicin	−	−	−
Sucrose	+	+	+
Raffinose	−	−	−
Rhomnose	−	−	−
Trehalose	+	+	+
Xylose	−	−	−
Filters			
Seitz	+	+	+
Selas 01	+	+	+
Selas 015	+	+	+
Selas 02	+	+	+
Selas 03	−	−	−

nary involvement was not noticeable. The lungs appeared normal at necropsy, and neither PPLO nor Pasteurella organisms were recovered from the lung tissues.

Calf 3 showed a rise of temperature up to 103.8 F. on the fourth day. The total erythrocyte and leukocyte counts were normal. On necropsy, three weeks later, the lungs were normal and no bacteria or PPLO were recovered.

DISCUSSION

Pleuropneumonia-like organisms have been involved in the pathogenesis of various diseases of man and animals.[2,3,7,11,13,15] The experimental results reported here showed that a pure culture of PPLO, alone or with *Past. multocida*, is not capable of producing the syndrome of shipping fever in calves. The organisms probably were potentially pathogenic and other synergistic agents were required for producing this condition.

Ryff and Gleen [14] reported the recovery of a viral agent from animals with shipping fever that produced hemagglutination of sheep and chicken erythrocytes. Such findings suggest that a virus of some nature probably is the primary etiological agent in shipping fever of cattle. Pleuropneumonia-like organisms and Pasteurella organisms may be secondary invaders in this disease. Pleuropneumonia-like organisms have a questionable role in diseases of various animals and birds and they are not completely understood.

Bovine PPLO were propagated successfully in chicken embryos. The organisms increased in virulence as the serial passage progressed. In the early transfers, the embryos were infected readily, but the mortality was low. When they were opened as late as 19 days after incubation, the embryos were involved grossly. At later passages, the embryos were dying four and five days after inoculation. The death pattern and the colony growth were similar to those of avian PPLO.[3,9] The biochemical reactions correlated well with those of avian strains.[8] It is possible that the bovine and avian strains have cross antigenic similarities. The ability of bovine strains of PPLO to pass through certain filters is generally similar to that of porcine PPLO.[15]

SUMMARY

Three strains of pleuropneumonia-like organisms (PPLO) were isolated from the respiratory tracts of calves with shipping fever. The range of pathogenicity of these three strains was studied in various laboratory animals. Hamsters, rabbits, and guinea pigs were refractory to all the strains. Antibodies against PPLO were demonstrated in turkey poults four weeks after intrasinus inoculation, but with no apparent sinusitis.

Exposure of 3 calves (with PPLO, a combination of PPLO and *Pasteurella multocida*, with or without stress) failed to reproduce the shipping fever syndrome.

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Effects of Endocrine Therapy on the Growth of Dwarf Beef Cattle

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DWARFISM has occurred in man and animals for many centuries. Until its occurrence was described in the three major breeds of beef cattle by Johnson *et al.*,[5] Baker *et al.*,[1,2] Gregory *et al.*,[4] and others, it was regarded by cattle breeders chiefly as a curiosity. It is now generally agreed that more than one type of dwarfism occurs in beef and dairy cattle, that dwarfs of the same general type do exhibit anatomical and physiological variation, and that the condition is heritable.

The most common type of dwarf beef calf, and the one which was observed in this study, has been referred to as an osteochondrodystrophic-like dwarf by Emmerson and Hazel.[3] This type of dwarf is small at birth, the forehead usually is bulging, and some degree of hydrocephalus exists. The tip of the tongue usually is visible, there is mismatching of the jaws, breathing is labored and noisy, and many cattlemen call the animals "snorter" dwarfs. With increasing age, the animals become pot-bellied and chronic bloat is a serious problem.

Because of the endocrine abnormalities which have been described in some human dwarfs, it was decided to determine the effects of estrogens, androgens, and the thyroid hormone on the growth of dwarf beef calves.

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MATERIALS AND METHODS

A total of 16 dwarf Hereford cattle of both sexes were observed over a period of more than one year. Nine were given endocrine therapy, and 7 were observed as controls. The animals were fed a grain ration of corn, oats, protein supplement, minerals, and mixed legume hay. They were allowed to run in a drylot during the winter and on a bluegrass pasture during the remainder of the year.

The thyroid hormone used was thyroactive iodinated protein and was administered daily in gelatin capsules with a balling gun. The androgen was implanted subcutaneously in the neck, in the form of compressed pellets containing 30 mg. of testosterone propionate each. The estrogen also

TABLE 1—Endocrine Therapy and Dosage Levels for Dwarf Beef Cattle

Dwarfs (No. and sex)	Age (days)	Duration of treatment (days)	Hormone dosage		
			Thyroprotein (Gm./100 lb. body wt. daily)	Testosterone propionate (mg.)	Diethylstilbestrol (mg.)
26 F	84	134	1.0	.....	.....
	218	105	1.5	.....	.....
	323	223	2.0	.....	.....
24 M	136	134	1.0	.....	.....
	270	105	1.5	.....	.....
	375	223	2.0	.....	.....
28 M	161	61	.....	30	.....
	222	178	.....	30	.....
	400	223	.....	60	.....
35 F	227	125	.....	30	.....
	352	223	.....	60	.....
31 F	233	178	.....	.....	24
	411	223	.....	.....	48
25 F	343	75	.....	.....	24
	418	178	.....	.....	24
	596	90	.....	.....	48
41 F	153	223	2.0	30	24
43 F	284	223	2.0	60	.....
42 M	289	85	2.0	.....	48

TABLE 2—The Effect of Endocrine Therapy on the Growth of Dwarf Cattle

Dwarf (No. and sex)	Length of treatment (days)	Initial			Final			Average daily gain (lb.)	Increase in height (cm.)
		Age (days)	Weight (lb.)	Height at withers (cm.)	Age (days)	Weight (lb.)	Height at withers (cm.)		
THYROPROTEIN									
26 F	462	84	77	57	546	394	85	0.68	28
24 M	462	136	206	71	598	616	92	0.88	21
TESTOSTERONE PROPIONATE									
28 M	462	161	155	64	623	579	89	0.91	25
35 F	348	227	276	78	575	375	87	0.28	9
DIETHYLSTILBESTROL									
31 F	401	233	244	75	634	510	90	0.66	15
25 F	343	343	211	72	686	420	80	0.60	8
COMBINATION OF HORMONES									
41 F	223	153	136	65	376	211	74	0.33	9
43 F	223	284	357	84	507	549	98	0.86	14
42 M	85	289	420	85	374	482	91	0.72	6
UNTREATED DWARF CATTLE									
34 M	45	71	145	68	116	126	70	0.00	2
36 F	44	56	111	62	100	115	63	0.07	1
37 F	36	45	111	63	81	123	63	0.33	0
44 M	516	113	185	70	629	582	86	0.77	16
45 M	466	25	88	57	491	500	79	0.90	22
46 M	145	172	202	64	317	286	80	0.58	16
47 M	219	168	141	61	387	235	72	0.43	11

was implanted subcutaneously, in the form of pellets containing 12 mg. of diethylstilbestrol each. The animals were reimplanted with either androgen or estrogen at varying intervals. The dosages and duration of each treatment are shown (table 1). Growth was expressed in terms of weight gains and height at withers.

RESULTS AND DISCUSSION

All the dwarf animals grew extremely slowly (table 2), and even when more than 18 months of age were only approximately 90 cm. in height. The maximum average daily gain observed over an extended period (462 days) was 0.91 lb. daily. This is an unusually good gain for a dwarf, but less than half the rate expected for normal beef cattle. However, the continual problem of chronic bloat in most dwarf animals makes it difficult to maintain a high feed intake and a uniform rate of gain.

Because of the small number of animals and the differences in age, sex, and treatment, comparisons of individual animals are of more value than group averages. When animals of comparable ages are compared (table 2), it seems possible that some of the hormone treatments may have increased the rate of gain and may possibly have increased slightly the height at withers. It was apparent to most observers that the general appearance of the dwarfs was improved by all the hormone treatments, that the animals were easier to keep on feed during treatment and appeared to have somewhat more subcutaneous fat (finish). Although 2 control dwarfs gained as well as the average treated dwarf, the rate of gain generally was improved by all hormone treatments. Dwarf 24, treated with thyroprotein for 462 days, was comparable to untreated dwarf 44 at the beginning of treatment. After being given thyroprotein for 462 days, he was 6 cm. taller at the withers. The fact that untreated dwarfs 44, 45, and 46 were 79 to 86 cm. in height at final ages of 317 to 629 days and that 3 of the treated dwarfs did not exceed 85 cm. would certainly not justify the conclusion that a consistent growth response was obtained with any endocrine treatment.

SUMMARY

The administration of thyroactive iodinated protein, testosterone propionate, and diethylstilbestrol, singly or in combination, appeared to produce a slight increase in rate of gain and a slight but questionable increase in height at withers of dwarf beef cattle. There is no evidence that the dwarfism syndrome can be corrected by endo-

crine therapy with the thyroid, estrogenic, or androgenic hormones.

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Studies on Urolithiasis. I. Experimental Production Associated with Feeding in Steers

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THE OCCURRENCE of urinary calculi in cattle in feedlots suggests that certain feeds induce a predisposition to calculus formation, and that a number of animals become affected simultaneously as a result of environmental stresses imposed upon animals already predisposed by the dietary regimen.

Newsom [9] listed a number of suspected causes of urinary calculi; among these were mineral imbalances and the hydrogen ion concentration in the urine. He also mentioned that the highest incidence is observed in winter.

Newsom *et al.*[10] concluded that phosphorus is not involved in calculi formation as a direct cause.

Lindley *et al.*[7] used a factorial arrangement to study several treatments simultaneously and reported that, under the conditions of their experiment, supplementation with K_2HPO_4 was associated with the highest incidence of calculi.

A quotation of H. Meckel von Helmstad (1856) by Lichtwitz [6] stipulated that two factors were essential for the production of calculi—first, an organic compound, mucous in character and, second, a supersaturated solution of petrifying salts. The importance of this observation is substantiated by Boyce *et al.*,[2] who found an increased amount of mucoproteins in the urine of patients with calculous disease.

Rubin and Howard [11] postulated that the mechanisms of bone formation and calculous formation in the urinary tract are similar. By the use of differential staining of actively calcifying bone, they made observations which they interpreted to indicate that mineral deposition occurs on active centers of a mucoprotein. Examinations of abnormal tissue calcification, tartar from teeth, and urinary calculi showed similar staining characteristics.

MATERIALS AND METHODS

The objective of this trial was to assess the effect of diet upon the predisposition to calculosis, measured by alterations in the mucoproteins in the urine. Three dietary factors were used: the ratio of concentrate to roughage, the level of calcium, and the level of phosphorus. Each factor was used at two levels. The concentrate:roughage factor was used at levels of 4:1 and 8:1; the calcium and phosphorus were used at the levels recommended by the National Research Council [8] and at three times those levels.

A factorial arrangement of treatments was used to measure the magnitude of the changes produced by these factors and their interactions. The general design was a randomized pen trial, with the restrictions that the weight distribution was similar among replicates and that all steers were individually fed. Four replicates were used for the eight possible treatment combinations.

Four additional steers were placed on the rations composed of the low levels of the three factors and supplemented with 20 mg. of stilbestrol per steer per day in the feed. This group was not considered in the statistical analysis of the trial.

The composition of the feed is given (table 1). The concentrates and mineral supplements were mixed and pelleted. The pellets and chopped alfalfa hay were individually weighed and fed. The animals were weighed at the start and finish of the trial.

Urine samples were collected once while the steers were on alfalfa hay and four times during the feeding period. The samples were an aliquot of a single voiding of urine.

Analysis of the urine was made for pH, calcium, and phosphorus. Mucoprotein excretion was measured as protein-bound hexosamine by the Elson-Morgan reaction, using an adaptation to urine of the procedure for serum described by Winzler.[12] The urine was fractionated into soluble and protein-bound hexosamine by precipitating with phosphotungstic acid (PTA) and determining hexosamine on both the supernatant fluid and the precipitate. The supernatant fluid fraction was aspirated from the precipitate into a clean centrifuge tube, acidified to about 3 N with concentrated HCl, hydrolyzed for two hours in boiling water, and diluted to a total volume of 25 ml. An aliquot was used for the hexosamine determination. The PTA precipitate fraction was washed with alcohol, 3 N HCl was added, and it was hydrolyzed similarly. The tubes were capped with marbles to prevent excessive evaporation. Care was taken to equalize the salt and acid concentrations, as described by Boas,[1] before carrying out the Elson-Morgan reaction. The evidence of the validity of the use of hexosamine as a quantitative measure of mucoproteins has been summarized by Winzler.[12]

RESULTS

The composition of representative bladder calculi is given in terms of ammonia,

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TABLE 1—Composition of the Treatment Rations

Concentrate to roughage	4/1				8/1			
Phosphorus	Normal*		High		Normal		High	
Calcium	Normal	High†	Normal	High	Normal	High	Normal	High
Ingredient (lb.)								
Chopped alfalfa hay	20.0	20.0	20.00	20.00	11.00	11.0	11.00	11.00
Corn	23.0	23.0	23.00	23.00	24.90	24.9	24.90	24.90
Barley	23.0	23.0	23.00	23.00	24.90	24.9	24.90	24.90
Milo	23.0	23.0	23.00	23.00	24.90	24.9	24.90	24.90
Cottonseed meal (41%, expel.)	6.0	6.0	6.00	6.00	8.00	8.0	8.00	8.00
Beet molasses	4.3	3.3	2.20	1.80	6.30	3.6	3.20	2.30
$CaCO_3$	0.2	2.1	.....	0.85	0.45	2.4	.....	1.15
NaH_2PO_4	.....	.....	3.00	.....	.....	.....	2.35	.....
$CaH_4(PO_4)_2$	.....	.....	0.45	3.15	.....	.....	0.50	3.25
Calculated content								
Concentrate/roughage	4/1	4/1	4/1	4/1	8/1	8/1	8/1	8/1
Calcium (Gm./lb.)	1.9	5.4	1.90	5.40	1.80	5.3	1.80	5.30
Phosphorus (Gm./lb.)	1.8	1.8	5.30	5.40	1.80	1.8	5.40	5.40

* Normal = equal to the NRC requirement; † high = equal to three times the NRC requirement.

protein nitrogen, and hexosamine (table 2).

A summary of the effects of the change from alfalfa to a fattening ration is presented (table 3).

The results for all quantitative measurements on the eight treatment groups are summarized (table 4).

TABLE 2—Composition of Calculi

Steer No.	Ammonia nitrogen (mg./Gm.)	Protein nitrogen (mg./Gm.)	Total hexosamine (μg./Gm.)
B11	.....	....	269
B32	0.33	5.4	303
B33	.....	....	338
B27	.....	6.7	420
B28	0.68	7.8	560
B26	.....	8.2	735

The results of the examinations of the bladders and kidneys for calculi of the eight treatment groups are presented (table 5). Each bladder was examined by opening it over a black tray and washing it with water. The kidneys were sliced longitudinally and the individual major and minor calices examined.

The ninth group, fed normal levels of calcium and phosphorus and supplemented with 20 mg. of stilbestrol, gained more rapidly and reached a greater final weight than similar animals in the rest of the trial. Four days before the end of the experiment, 1 steer from this group was slaughtered because of straining and inability to urinate, and the urethra was found to be occluded with calculi. After slaughter, 2 other steers in this group were found to have both bladder and renal calculi. The fourth steer had no detectable calculi.

DISCUSSION

An hypothesis is made in this study of urolithiasis that the urinary mucoprotein excretion measured by the protein-bound hexosamine is a measure of predisposition to calculosis in animals. It is shown (table 2) that the calculi recovered during this trial contained considerably more protein nitrogen than ammonia nitrogen. This level of protein nitrogen is equivalent to about 3 to 5 per cent protein (protein nitrogen $\times$ 6.25). The amounts of hexosamine are proportional to the protein nitrogen. This suggests the occurrence of a mucoprotein

TABLE 3—Summary of the Effects on Urine Composition of Shifting from an Alfalfa Ration to a Concentrate

Item	Units	Average while on alfalfa	Average effect of concentrated ration	Probability
Calcium	mg./100 ml.	8.30	−3.61	<0.005
Phosphorus	mg./100 ml.	51.20	+28.49	<0.005
Soluble hexosamine	μg./ml.	38.00	+51.13	<0.005
Protein-bound hexosamine	μg./ml.	0.75	+2.45	<0.005

TABLE 4—Summary of the Factorial Treatment Effects

Item	Control	Main effects			Interactions†			
		Ratio*	Ca	P**	Ratio and Ca	Ratio and P	Ca and P	Ratio and Ca and P
Bladder calculi (Gm.)	2.50	−0.52	−0.01	−1.28	+0.20	+0.38	+0.19	−0.30
P**	.....	.....	.....	<0.05	.....	.....	.....	.....
Carcass weight (lb.)	560	+2.69	+1.19	−4.81	+6.69	+1.19	+9.31	−25.69
P	.....	.....	.....	.....	.....	.....	.....	<0.05
Weight gain (lb.)	524	−12.19	+14.06	−3.34	+3.34	+8.44	+18.44	−27.19
P	.....	.....	.....	.....	.....	.....	.....	=0.10
Urine pH	7.66	−0.26	+0.12	−0.46	−0.06	−0.01	0.0	+0.21
P	.....	<0.05	.....	<0.005	.....	.....	.....	.....
Urine calcium (mg./100 ml.)	5.98	+0.97	−0.23	−1.29	+0.67	−0.59	+1.16	−0.79
P	.....	.....	.....	.....	.....	.....	.....	.....
Urine phosphorus (mg./100 ml.)	66.25	+10.51	+7.20	+16.29	+8.17	−5.03	+3.01	+1.00
P	.....	<0.05	<0.10	<0.005	<0.05	.....	.....	.....
Soluble hexosamine (μg./ml.)	100.00	+6.47	+10.25	−1.18	+11.69	+1.84	+15.12	−8.28
P	.....	.....	.....	.....	.....	.....	=0.05	.....
Protein-bound hexosamine (μg./ml.)	3.31	+0.81	+1.16	+0.55	+1.07	+0.29	+0.81	−0.55
P	.....	<0.05	<0.01	.....	<0.01	.....	<0.05	.....

* Ratio indicates the ratio of concentrate to roughage; † these are the differences of the averages on the high level compared with those of the low level; ** P = probability.

The values given in the control column are the averages for the groups on the low levels of the three factors. The values for the main effects and interactions are the average effects of increasing a factor to the higher level in the ration, and the sign indicates the direction of the effect. The probability (P) of a random occurrence of an equal value or greater is given in the column directly below each value; leaders (.....) indicate no significance at a level of one chance in ten or less.

matrix within the calculi. Boyce and Sulkin [3] have described the matrix portion of calculi in detail and have demonstrated its mucoprotein character. There seems to be a reasonable probability that the mucoproteins appearing in the urine are the source of the mucoproteins appearing in the calculous matrix. It is also probable that these mucoproteins have an active role in mineral deposition in the urinary tract, as suggested by Rubin and Howard.[11]

The effect of the shift from alfalfa hay to the fattening ration on the composition of the urine is apparent (table 3). This shift produced a highly significant ($P < 0.005$) increase in the urinary output of protein-bound hexosamine, suggesting that increasing the amount of concentrate fed increases the predisposition to calculosis. The increased output of phosphorus ($P < 0.005$ is due largely to its increased intake and the reduced output of calcium ($P < 0.005$) to its reduced intake. There seems to be little significance in the values for the soluble hexosamine.

A number of significant effects of the three dietary treatments are evident (table 4). The increase of the concentrate to roughage ratio from 4:1 to 8:1 increased the urinary output of mucoproteins significantly ($P < 0.05$). The effect probably is related to the change observed when the animals were shifted from roughage to a concentrate, and substantiates the effect on the urinary protein-bound hexosamine of increasing the concentrates fed. The mechanism of this action is obscure and will probably remain so until more is known about the origin of the muco-complexes in the urine. The high level of the concentrate to roughage ratio was also accompanied by an increase in the phosphorus in the urine ($P < 0.05$) and, since the rations contained a similar distribution of the amounts of phosphorus, this suggests an increased turnover rate of the tissues.

TABLE 5—Calculi Recovered from Urinary Tracts

Concentrate to roughage	4/1				8/1			
Phosphorus	Normal*		High		Normal		High	
Calcium	Normal	High†	Normal	High	Normal	High	Normal	High
Total bladder calculi/Gm.	9.00	6.16	0.39	1.50	3.33	5.33	0.23	0.53
Bladder containing calculi	4	4	1	3	4	4	4	4
Kidneys containing calculi	3	3	2	4	4	4	3	2

* Normal = equal to the NRC requirement; † high = equal to three times the NRC requirement.

The change in phosphorus excretion accounts for the acidification of the urine ($P < 0.05$) also observed.

Increasing the level of calcium to three times the National Research Council allowances did not increase the output of calcium in the urine, but was accompanied by a highly significant ($P < 0.01$) increase in the urinary protein-bound hexosamine. The two-way interactions containing the high level of calcium also increased the output of protein-bound hexosamine significantly. It is generally accepted that calcium absorption from the digestive tract is proportional to the level in the ration, particularly in growing animals. Such calcium can be absorbed on skeletal surfaces, incorporated into the bone structure, or excreted in the urine. In the past, excretion of excess calcium into the large intestine was generally accepted, but recent isotope studies [5] indicate that endogenous calcium is derived from the intestinal secretions. Since, in this trial, there was no increase in the amount of calcium in the urine, greater absorption due to any increased level of feeding probably was incorporated into the skeletal structure. The incorporation of the calcium into the structure of bone is accompanied by an increased turnover of the mucopolysaccharide in the matrix of bone,[4] hence the effect of the high level of calcium on the output of protein-bound hexosamine is indicative that the matrix of bone is one source of the muco-complexes appearing in the urine.

The role of the phosphate ion in the production of calculi has never been clear, for it has been stated that phosphorus is not involved [9] and that its level in the ration is associated with the incidence of calculi.[7] In this trial, its most obvious association was with a reduction in the amounts of bladder calculi. Increasing the level of the phosphate ion did not alter the level of protein-bound hexosamine, but did result in an acidification of the urine ($P < 0.005$) and in a reduction in the amounts of bladder calculi ($P < 0.05$). There is probably a cause and effect relation between the acidification of the urine and the reduction in bladder calculi.

These results suggest that the phosphate ion is not involved directly with the mechanism that promotes mineral deposition, as measured by the amount of protein-bound hexosamine in the urine. It appears to reduce the probability of the occurrence of calculi by acidification of the urine, and also serves as a target substance of the mechanism promoting mineral deposition, resulting in the formation of phosphatic calculi. This latter effect may account for its frequent association as a cause of calculosis.

As stated, there is little significance in the values for soluble hexosamine in the supernatant fraction of the urine after precipitation with phosphotungstic acid in the urine. Another significant effect observed, which probably bears no relation to the problem at hand, is the reduction in final carcass weight that occurred in the presence of the high level of all three factors.

The distribution of bladder and renal calculi among the eight treatment groups is summarized (table 5). There is no association of treatments with the occurrence except that with the high level of phosphorus. The incidence is high and it appears that the ration is useful for the experimental production of calculi. There is some component in the ration increasing the tendency toward calculi formation, and it appears that it is most closely associated with the concentrate portion of the ration (tables 3, 4). It seems doubtful that mineral imbalances have an important role in the occurrence of this predisposition, and the phosphate ion is only secondarily involved. The results suggest that too much emphasis has been placed upon the mineral composition of calculi and that a more informative approach is the study of the predisposition to calculosis measured by the urinary output of muco-complexes.

SUMMARY

The quantity of protein-bound hexosamine occurring in the urine of steers was measured by the hexosamine content of the precipitate formed in urine by phosphotungstic acid.

Significant increases in the amounts of protein-bound hexosamine in the urine were shown to occur when the steers were shifted from an alfalfa hay ration to a fattening ration, when the concentrate to roughage ratio was increased from 4:1 to 8:1, and when the level of calcium in the ration was increased to three times the National Research Council requirements.

The two-way interactions, involving the high levels of concentrate to roughage with the calcium and calcium with phosphorus,

produced significant increases in the urinary mucoproteins.

The high level of phosphorus produced a highly significant acidification of the urine and significant reduction in the amounts of calculi recovered in the bladders, but had no effect upon the output of protein-bound hexosamine in the urine.

An hypothesis is made that predisposition to calculosis can be measured by the level of mucoproteins in the urine.

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The Effect of Chlortetracycline, Fed Continuously During Fattening, on the Incidence of Liver Abscesses in Beef Cattle

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ABSCESSED LIVER, an infectious, noncontagious disease of cattle, is asymptomatic except in extreme cases and is caused by *Spherophorus necrophorus*. The disease is encountered in approximately 5 per cent of cattle slaughtered in the United States. A high incidence may occur in some lots of fattened cattle. All abscessed livers are condemned at slaughter as unwholesome food.

This investigation was conducted to determine the effect of chlortetracycline (Aureomycin*), fed continuously at low level during the fattening period, on the incidence of liver abscesses in beef cattle.

REVIEW OF LITERATURE

Newsom [7] and Madin [5] identified *S. necrophorus* as a pure infection in approximately 85 per cent of a large sample of abscessed livers from beef cattle. An additional 10 per cent contained *S. necrophorus*, plus other contaminating organisms. Jensen *et al.*[4] experimentally reproduced liver abscesses by inoculating pure cultures of *S. necrophorus* into the portal veins of cattle.

Smith [8] and Jensen *et al.*[3] studied the stomachs and livers of fattened cattle at slaughter and determined a positive correlation for the concurrence of rumenitis and liver abscesses. The entrance of *S. necrophorus* from ruminal ingesta into the ruminal wall and the subsequent entrance into the portal system through foci of rumenitis were postulated.

Matshushima *et al.*[6] studied the effect of chlortetracycline given to baby calves on the incidence of liver abscesses at slaughter of the same fattened animals at approximately 1 year of age. In each of two experiments, 5 calves were fed chlortetracycline daily during the first 12 weeks of life, while 5 control calves were untreated. After weaning at 7 months of age and fattening in a feedlot to a body weight of approximately 1,000 lb., the cattle which had been given the antibiotic orally during the first 12 weeks of life had significantly fewer abscessed livers than did the cattle which were given no antibiotic during this time.

In 1957, Bowman *et al.*[1] reported the effects of feeding animal fat, stilbestrol, and chlortetracycline to 50 fattening cattle. They found that "Chlortetracycline decreased the incidence of liver abscesses ($P < 0.05$)."

MATERIALS AND METHODS

Three experiments were conducted over a period of two years, two in 1956 and one in 1957, in commercial feedlots near Sterling, Colo.

Experiment 56A.—A group of 593 heifers from northern Texas, weighing approximately 600 lb. each, were assigned at random by truck loads to form two lots. Lot 1 was given no antibiotic-containing ration, while lot 2 was fed a ration that contained enough antibiotic to furnish 75 mg. of chlortetracycline per heifer per day during the fattening period of 120 days.

The ration consisted of alfalfa hay, 16 lb.; millet, 8 lb.; ground corn, 5 lb.; barley, 4 lb., soybean meal, ⅔ lb.; alfalfa pellets, 1 lb.; stilbestrol, 10 mg.; and molasses, 1 lb.

Experiment 56B.—Another group of 399 similar heifers also were assigned at random by truck loads to form two lots. Lot 3 was given no antibiotic, and lot 4 was fed 75 mg. of chlortetracycline per heifer per day for 120 days. The ration consisted of alfalfa hay, 4 lb.; cottonseed cake, 1 lb.; millet, 2 lb.; ground corn, 14 lb.; stilbestrol, 10 mg.; and molasses, 1 lb.

Experiment 57A.—A third group of 903 similar heifers were assigned at random by truck loads into three lots (10, 11, 14). They were fed a standard ration which did not vary essentially from that fed to those in experiment 56B. The feeding period was 117 to 133 days.

Lot 11 was given no antibiotic, lot 10 was given 70 mg. of chlortetracycline per animal per day throughout the feeding period, and lot 14 was fed the same antibiotic supplement plus 1.8 Gm. of tetra-alkylammonium stearate (Dynafac*) per heifer per day during the fattening period.

The 1956 experiments were conducted during the winter months; the 1957 experiment was started in July and ended in November.

At slaughter, the livers of all cattle were examined carefully for abscesses and all data were analyzed statistically.

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This investigation was supported in part by grants from the American Cyanamid Co., Princeton, N.J., and from meat packing companies in Denver, Colo.

* Aureomycin is produced by the American Cyanamid Co., Princeton, N.J.

* Dynafac is produced by Armour and Co., Kankakee, Ill.

RESULTS

In each of the three experiments, the feeding of chlortetracycline decreased significantly the number of liver abscesses (table 1).

The amount of feed required for 100 lb. of gain in body weight was 1,005.2 lb. for control animals in lot 11, 926.7 lb. for animals in lot 10 which were fed chlortetracycline as a supplement, and 939.3 lb. for animals in lot 14 which were given chlortetracycline and tetra-alkylammonium stearate as a supplement.

TABLE 1—Effect of Chlortetracycline on Incidence of Liver Abscesses in 1,895 Fat Cattle

Experiment	Year	Lot (No.)	No. of cattle	Chlortetracycline (mg./heifer/day)	Abscesses (No.)	Abscesses (%)	Probability
56A	1956	1	291	none	95	32.64	$P < 0.05$
		2	302	75	76	25.15	
56B	1956	3	199	none	80	40.02	$P < 0.01$
		4	200	75	8	4.00	
57A	1957	11	224	none	154	68.75	$P < 0.01$
		10	389	70	53	13.62	
		14	290	70*	56	19.31	

* Plus 1.8 Gm. of tetra-alkylammonium stearate per animal per day.

DISCUSSION

The incidence of bovine liver abscesses is higher in the western part of the United States than in the midwestern and eastern parts. About 13 per cent of all cattle killed at Denver, Colo., packing establishments have abscessed livers, according to Animal Research Service data. For the country as a whole, condemnations of bovine livers due to abscesses average about 5 per cent. The financial loss to the livestock industry obviously is a major one, and an economically sound procedure to reduce this loss would mean more profit to those engaged in fattening and processing cattle.

Under the conditions of this experiment, which involved 1,895 cattle, the continuous feeding of chlortetracycline significantly reduced the incidence of liver abscesses and at the same time resulted in increased feeding efficiency.

The cause of these benefits has not been established, but has been postulated to be due to such factors as: (1) more normal liver function, due to reduction in abscesses; (2) changes in flora of the digestive tract which are beneficial to the animal; and (3) a general increase in resistance of the animal to clinical and subclinical infections.

The beneficial effects of adding antibiotics to feed rations have been studied more extensively in nonruminant animals and chickens. Recently, the use of antibiotic supplements in cattle and sheep feed has increased, adding emphasis to the need for more detailed studies in ruminants.

Cunha [2] believes that "Antibiotics have their greatest effect when fed to calves and steers with low grade infections brought about by conditions of stress, such as shipping exposure, feedlot infection, bad weather and poor management." Referring more specifically to lambs, he states that "the biggest reason for antibiotics (in the feed) is its suppressing effect on feedlot infections."

These conclusions may be supported by the infection encountered in this study. Approximately 30 days after the 1957 experimental cattle were put on feed, infectious bovine rhinotracheitis developed. The feedlot operator reported a much higher incidence of disease among the controls than among the animals given chlortetracycline in their ration. He found the disease to be more severe in the control pen, and said sick animals required more treatment than those receiving the antibiotic.

No evidence exists that this antibiotic has a viricidal effect, but much of the distress of rhinotracheitis appears to be due to secondary bacterial invaders, and it is possible that the antibiotic was beneficial in this respect.

Many cattle feeders routinely feed antibiotics as a means of lowering the cost of gains, so the reduction in incidence of liver abscesses costs nothing extra.

SUMMARY

Three experiments, using 1,895 cattle, were conducted to determine the effect of chlortetracycline, fed continuously at the

rate of 70 or 75 mg. per animal per day throughout the fattening period, on the incidence of liver abscesses. In each experiment, the cattle given chlortetracycline developed significantly fewer abscesses than untreated cattle.

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The Effect of Adrenocorticotrophic Hormone on the Circulating Eosinophil Level—A Possible Screening Test for Adrenal Gland Function in the Cow

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To ESTABLISH the role of the adrenal gland in a disease process, a reliable test to evaluate its function must be found. The most reliable evidence of an adrenal cortical insufficiency is the demonstration of a decrease in the level of adrenal cortical hormone in the blood or urine, or both. There have been few reports on the determination of the level of corticoids in cattle[2] and, under the conditions of large animal practice, it is often difficult to obtain such data. For this reason, a simple test that might be used as an aid in diagnosing adrenal cortical insufficiency in cattle should be of practical value.

The hormones secreted by the adrenal cortex have a variety of actions. One consistent effect is on the circulating eosinophil level. Any increase in the level of the cortisone-type adrenal cortical steroids (*i.e.*, the 11- and 11-17-oxysteroids) results in a marked eosinopenia. This effect has been demonstrated in man,[1,8] mice,[7] and dogs.[3] Thus, if a dose of adrenocorticotrophic hormone (ACTH) sufficient to cause secretion of cortical hormone is administered, the level of circulating eosinophils falls. This response depends on the adrenal cortex being in a functional state and, if no decrease in eosinophils is observed, it may be presumed that an adrenal cortical insufficiency exists. Using the circulating eosinophil level as a criterion, a test has been developed by Thorn *et al.*[8] for use in human beings, to evaluate adrenal gland function. Martin *et al.*[3] proposed a similar test as a screening method for evaluating adrenal cortical function in the dog. This report describes the effect of injections of ACTH on the number of circulating eosinophils in cattle.

MATERIALS AND METHODS

The jugular or mammary vein was used to collect blood samples, and powdered heparin was used as the anticoagulant. Before drawing the blood sample into the leukocyte pipette, the sample was inverted several times to insure proper mixing. The sample was drawn up to the 1.0 mark of the pipette; a special diluting fluid then was drawn up to the 11 mark of the pipette.

The diluting fluid, as described by Pilot,[5] is prepared as follows:

Propylene glycol	50 ml.
Distilled water	40 ml.
Phloxine (1%) in distilled water (stock solution)	10 ml.
Sodium carbonate (10%)	1 ml.

These ingredients, with the exception of the 10 per cent sodium carbonate solution, may be prepared as stock solutions. The completed solution is reported to be stable for at least one month but, in most of the trials reported here, it was not used for longer than one week.

The pipette containing blood and diluting fluid then was shaken vigorously on a mechanical shaker for about two minutes. After shaking, four or five drops of the diluted blood were discarded from the pipette. The counting chambers of the hemacytometer were filled and allowed to remain for 15 minutes in a Petri dish containing moist cotton pledgets to prevent evaporation from the counting chambers. The stained eosinophils in each chamber then were counted.

Two pipettes were prepared by each experimenter as described, and each pipette was used to fill two counting chambers, resulting in a total of eight counting chambers being filled for each sample of blood. The eosinophils within all nine squares of each chamber were counted. The average of the eight chamber counts was taken as a factor which, when multiplied by 11.1, gave the number of eosinophils per cubic millimeter of blood. The percentage of change in the eosinophil count in blood samples taken after ACTH was given was computed, using the following formula:

$$100 - \frac{\text{eosinophils/cmm. after ACTH}}{\text{eosinophils/cmm. before ACTH}} \times 100$$

The initial blood sample was drawn and, immediately afterward, ACTH was injected intramuscularly. All injections, which varied from 150 to 600 units, were given either in the early morning or late evening. The ACTH was in a gelatin vehicle.

The initial blood sample was counted according

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to the method described. Blood samples were collected at each succeeding hour and the eosinophils counted in a similar way. This was continued until the eosinophil count dropped to its lowest level and then began to rise. Other similar trials were performed, using varying doses of ACTH (150, 250, 300, 310, 400, and 600 units), until the minimum dose giving the maximum decrease in circulating eosinophils was established. This dose, which we refer to as the "practical minimum dose," was used in all succeeding trials in different animals. Since the practical minimum dose to be established was intended for all animals, regardless of their weights, it was felt that a 400-unit dose would elicit a consistent response in animals of varying body weights. This dose was used, rather than lower doses which caused a maximum decrease only in lighter weight animals.

It was observed that the maximum decrease occurred at or about the tenth hour in each trial. Therefore, as the experiment progressed, the last postinjection count was made at approximately the tenth hour. In some trials, blood was refrigerated and allowed to stand before counting eosinophils.* Eosinophil counts made on eight blood samples immediately after collection and then from three to 15 hours after refrigeration indicated that no significant change occurred as a result of refrigeration. This indicates that bovine eosinophils are more stable than those of the dog.[4] In this species, it is necessary to make direct eosinophil counts immediately after obtaining blood samples.

When the practical minimum dose and the time at which the maximum decrease in circulating eosinophils occurred were established, trials were performed on cows selected at random. These included both normal and diseased animals. Control trials were performed on 3 of these animals.

* Four and one half hours was the maximum time that blood was allowed to stand.

RESULTS

A total of 24 trials was performed, using 11 animals (table 1). Doses of ACTH varied from 150 to 600 units, and counts were made from the first to the nineteenth hour, inclusive. The practical minimum dose which gave the maximum decrease in circulating eosinophils was 400 units. The time at which this decrease occurred was ten hours after injection of the ACTH. In all trials where 400 units was used, the decrease in the circulating eosinophil level was greater than 65 per cent. With the dosage being constant, there appeared to be a relationship between weight and the percentage of decrease in the circulating

TABLE 1—Response of Circulating Eosinophils in Cows to Varying Doses of Adrenocorticotrophic Hormone

Animal No.	Weight (lb.)	Experiment No.	Dose (units)	Eosinophil count (cells/cmm.) Before ACTH	Eosinophil count (cells/cmm.) After ACTH	Hours after injection*	Decrease of eosinophils (%)
1	.....	1	150	244	89	9.0	64
2	900	2	150	733	355	6.0	52
2	900	3	250	1,388	511	7.0	65
2	900	4	600	1,243	111	11.0	91
2	900	5	400	588	56	9.5	91
2	900	6	310	444	178	9.5	60
3	1,060	7	400	355	56	9.0	75
3	1,060	8	300	644	133	9.5	79
4	.....	9	400	844	56	9.5	93
2	900	10	400	1,321	200	9.5	85
3	1,060	11	300	477	133	9.0	72
5	.....	12	400	666	100	10.0	85
6	.....	13	400	289	44	10.0	85
4	.....	14	400	1,199	100	10.0	92
2	900	15	400	1,055	233	11.0	78
3	1,060	16	400	344	56	12.0	84
7	800	17	400	389	33	10.0	91
8	1,000	18	400	1,110	355	10.0	67
9	990	19	400	899	144	10.0	84
10	1,325	20	400	988	333	10.0	66
11	850	21	400	566	78	10.0	86
5	.....	22	Sterile water (20 ml.)	834	677	10.0	20
4	.....	23	Sterile water (20 ml.)	1,067	888	10.0	18
2	900	24	Sterile water (20 ml.)	699	612	10.0	11

* Hour at which the post-treatment eosinophil count was made.

Animals 1, 2, 3, 4, 7, 9, and 11 were clinically normal; animal 5 was an undiagnosed clinic case; animal 6 had actinomycosis and was given radiation therapy 36 hours before the trial; and animals 8 and 10 had hemolytic micrococcic mastitis.

eosinophil level; *i.e.*, heavier animals required larger doses.

The initial eosinophil counts varied markedly between animals and between different trials on the same animal. This appears to be true in both man [8] and the dog.[3] It was observed that the initial eosinophil count did not influence the percentage of decrease of the circulating eosinophil level after administration of ACTH.

DISCUSSION

The results reported are intended only as a preliminary step in the development of a clinical test for diagnosing adrenal cortical insufficiency in cows. The results of this test should be correlated with the clinical signs of animals suspected of having an insufficiency and, if possible, the levels of corticoids in blood or urine, or both, should be determined. To establish this as a reliable test, it will be necessary to (1) determine the effect of ACTH on eosinophil levels in cows with known adrenal insufficiency; (2) correlate blood and urine corticosteroid levels with the eosinophil responses in normal cows and cows with adrenal insufficiencies (since adrenal insufficiency has not yet been proved a disease entity, it would be necessary to use adrenalectomized animals to evaluate this test further); and (3) study a large number of animals with diseases of known cause to determine any effects on the response of circulating eosinophils to ACTH.

Since the initial eosinophil count does not affect the percentage of decrease in circulating eosinophils after administration of ACTH, diurnal variation, if it occurs in cows, has no effect on this percentage of decrease.

SUMMARY

The effect of intramuscular injections of adrenocorticotrophic hormone (ACTH) on the level of circulating eosinophils in normal cows was studied. After the injection of a 400-unit dose, eosinophils decreased by 66 to 93 per cent, as compared with the preinjection level. The maximum decrease occurred approximately ten hours after injection. It is suggested that the determination of the percentage of decrease in circulating eosinophils after the administration of ACTH may be of value as a screening test for adrenal cortical function in cows.

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Cholinesterase Activity of Bovine Blood

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THE AVERAGE VALUE and range of erythrocyte cholinesterase activity of both cattle and sheep were established in a recent paper by Radeleff and Woodard.[8] A large number of animals was used (253 cattle, 180 sheep). The method used was that of Michel,[3] an electrometric method in which the cholinesterase activity is expressed as a change in pH units per hour. According to this method, acetic acid is liberated from the acetylcholine substrate by the activity of the cholinesterase in the erythrocyte sample. The liberated acid causes a change in the pH of the buffer solution in which the reaction proceeds. The rate of change of pH is related to the amount of cholinesterase available to hydrolyze the acetylcholine substrate.

The method of Michel probably has been used more than any other single method for the determination of blood cholinesterase activity. However, it has certain limitations. This technique was designed for use with blood that has been refrigerated and anticoagulated with heparin, and in the absence of hemolysis. These three conditions can not always be satisfied. This is particularly true when blood samples are collected under markedly varying, unpredictable conditions such as might obtain in rural areas where refrigeration may not be available. Even if the blood samples are refrigerated upon collection, they might have to be transported to the laboratory without adequate cooling. This is particularly true of samples sent by mail. In these instances, lack of refrigeration and shaking may combine to cause hemolysis, which contributes to errors in the determination of cholinesterase activity by this method. With hemolyzed blood, there is a decrease in the erythrocyte level and an increase in the plasma cholinesterase level.

Furthermore, heparin, although commonly available, is not always to be found. Citrate and oxalate are used widely as anticoagulants. There is no assurance that heparin will be or has been used as the anticoagulant in any given instance.

Because of these limitations, it would seem that other methods of blood cholinesterase analysis might be used to advantage. Consequently, the method of Fleisher and Pope[1] was investigated. This method depends on the formation of a colored compound from acetylcholine substrate in the experimental situation following the action of the cholinesterase. Since this method is based on a series of specific chemical reactions and the colorimetric determination of a colored compound, it is precise and reproducible. In our hands, the method does not yield different results when various anticoagulants are used or when the blood sample is stored at room temperature. It does not require more of the analyst's time than the method of Michel and it is no more difficult to carry out.

The original procedure of Fleisher and Pope[1] has been slightly modified and, in some respects, our modifications resemble the later method of Fleisher *et al.*[2] The most important variation is the adaptation of the method for erythrocyte cholinesterase determination, rather than whole blood cholinesterase.

MATERIALS AND METHODS

The eryturocyte cholinesterase was determined in two groups of cattle. The first was a group of 30 cattle of various breeds, sex, and ages, being slaughtered for market. The blood was obtained at the time of slaughter as the jugular veins were incised. The addition of an anticoagulant was unnecessary, for the blood remained fluid after collection. The reasons for this postmortem fluidity of the blood are unknown.[4] The presence or absence of an anticoagulant did not affect the results.

A second group consisted of 27 Aberdeen Angus cattle of different ages and sexes. Blood was collected from the jugular veins of these animals and was prevented from coagulating by using sodium citrate. All blood from both groups of cattle was refrigerated in the interval between collection and analysis. The cholinesterase determinations were performed within six days of collection.

Each blood sample was centrifuged at 3,000

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r.p.m. for 15 minutes. The plasma was decanted and physiological saline solution was added to make an artificial hematocrit measurement of 50 per cent. The well-mixed erythrocyte and saline solution sample then was analyzed according to the method of Fleisher and Pope, with our modifications.

Our modified method is only outlined here; a detailed methodology is available from the author.[6]

The following solutions used in the analysis were essentially those of Fleisher and Pope:

Solution A.—Phosphate buffer, 0.134 M, pH 7.2, was prepared by mixing 3 parts by volume of a solution containing 23.5 Gm. of anhydrous Na_2HPO_4 per liter with 1 part of a solution containing 18.2 Gm. of KH_2PO_4 per liter; the pH is adjusted to 7.2 if necessary. Buffer and stock solutions must be refrigerated when not in use.

Solution B.—Acetate buffer, 0.001 M, pH 4.5 (100 ml.), as required for solution C, may be prepared by mixing 42 ml. of 0.2 N sodium acetate (27.22 Gm./liter) with 58 ml. of 0.2 N acetic acid (11.3 ml./liter). This solution must be refrigerated when not in use.

Solution C.—Acetylcholine, 0.04 M, is prepared with 0.7266 Gm. of acetylcholine chloride (Merck) in 100 ml. of 0.001 M acetate buffer (solution B). This must be refrigerated when not in use and should not be used if over 3 weeks old.

Solution D.—Acetylcholine, 0.004 M, is 1 volume of solution C diluted with 9 volumes of solution A. It is made daily as required.

Solution E.—Hydroxylamine hydrochloride, 2 M, is made with 27.8 Gm. of hydroxylamine hydrochloride dissolved in distilled water to 200 ml. It must be refrigerated when not in use and should not be used if over 2 weeks old.

Solution F.—Sodium hydroxide, 3.5 M, is made with 28 Gm. of sodium hydroxide dissolved in distilled water to 200 ml.

Solution G.—Alkaline hydroxylamine consists of equal volumes of solutions E and F, mixed shortly before use in a quantity required for the samples being analyzed. It should not be used if it is over 30 minutes old.

Solution H.—For hydrochloric acid, use concentrated acid, specific gravity 1.18, diluted with 2 volumes of distilled water.

Solution I.—For hydrochloric acid, 0.1 N, use 8.3 ml. of concentrated HCl (solution H) per liter.

Solution J.—Ferric chloride, 0.37 M, is made with 10 Gm. $FeCl_3 \cdot 6H_2O$ made up to 100 ml. with solution I.

Solution K.—Saponin solution, 0.01 per cent, consists of 0.1 Gm. of solution per liter of distilled water.

Solution L.—Sodium chloride, isotonic, is made with 9 Gm. of sodium chloride per liter of distilled water.

Ordinary laboratory equipment, 15-ml. centrifuge tubes, 50-μl. (microliter) pipettes, and a colorimeter or spectrophotometer are needed.

Fifty microliters of the diluted (50% hematocrit) erythrocyte solution was rinsed into 0.95 ml. of saponin solution (solution K). A second identical sample was prepared and set aside for the control determination. At zero time, 1 ml. of solution D was added to the first sample, and the contents of the tube were mixed and incubated at 25 C. A control tube of 1.0 ml. of solution D only also was incubated. At the end of ten minutes, 4 ml. of solution G was added to each tube. Both tubes were shaken well. After one minute, the control erythrocyte solution sample was added to the control tube of solution K. Immediately, 2 ml. of solution H was added to each tube, followed rapidly by 2 ml. of solution J. Thorough mixing was carried out after each solution was added. The solutions in the tubes then were filtered through Whatman No. 40 filter paper and readings were made in the Klett-Summerson photoelectric colorimeter, with filter 54, having been calibrated previously to zero with a distilled water blank. (If the Coleman junior spectrophotometer is used, the wave length is set at 540 μ.)

To calculate the number of micromoles (μM) of acetylcholine which disappeared during incubation (the true cholinesterase activity), the following formula was used:

No. μM of acetylcholine utilized by the erythrocyte (true) cholinesterase =

$$4\left(1-\frac{Re}{Re}\right)$$

(Re = colorimetric reading of experimental sample; Re = colorimeter reading of control sample.)

RESULTS AND DISCUSSION

The erythrocyte cholinesterase activity of the two groups of cattle is reported (table 1). No significant variation was observed when the type of anticoagulant

TABLE 1—Erythrocyte Cholinesterase Activity (μM of Acetylcholine Hydrolyzed) of Bovine Blood

Group	No. cattle	Average	Maximum	Minimum	s.d.*	Range (av. ± s.d.)
Unselected slaughterhouse cattle	30	1.26	2.14	0.50	0.34	0.92–1.60
Purebred cattle	27	1.62	2.25	1.10	0.27	1.35–1.89

* Standard deviation.

was changed, or when the blood was not refrigerated.[7]

The erythrocyte cholinesterase activity of the two groups of cattle was significantly different. The average value for erythrocyte cholinesterase activity in unselected cattle was 1.26 μM of acetylcholine hydrolyzed, with a range of 0.50 to 2.14 and a standard deviation of 0.34. The average value for Aberdeen Angus cattle was 1.62 μM, with a range of 1.10 to 2.25 and a standard deviation of 0.27. It is of interest that the unselected group had a lower average erythrocyte cholinesterase activity, a larger standard deviation, and a greater range of values than did the group of carefully maintained purebred Aberdeen Angus cattle. The reason for this is not immediately apparent. No comparative studies of erythrocyte cholinesterase activity of various breeds of cattle have been published insofar as the authors are aware. There is no real reason to suspect that various breeds of cattle would have different levels of erythrocyte cholinesterase activity.

The remote possibility exists that some of the unselected slaughterhouse cattle had been exposed to the organic phosphate insecticides. As contact with these anticholinesterase insecticides would cause a depression of erythrocyte cholinesterase activity, this is a possible explanation of the lower average value and the greater range of cholinesterase activity in this group of cattle. Such exposure might have occurred in several ways—by dipping or spraying, by feeding on forage contaminated by residuals of the organic phosphates, or by having been exposed to drifting clouds of the organic phosphates being used nearby It is impossible to do more than speculate on the possible exposure of these unselected cattle to the organic phosphates. In the case of the Aberdeen Angus cattle, the herd was maintained in areas known to be free of contamination with the organic phosphates.

The erythrocyte cholinesterase activity in man is remarkably stable in any given individual and there is no reason to expect more variation in other animals. The state of nutrition, various drugs, liver diseases, and other factors have been shown to affect the plasma cholinesterase levels in man,[5] but do not change the erythrocyte cholinesterase levels.[10] Immature erythrocytes have a greater cholinesterase content than do mature erythrocytes. Therefore, in recovery from certain anemias, the erythrocyte cholinesterase activity may be increased above normal; the measurement of erythrocyte cholinesterase activity has been proposed as one method of following recovery from anemia.[9] However, to our knowledge, the only way in which the erythrocyte cholinesterase activity may be effectively depressed is by exposure to the anticholinesterase organic phosphate compounds.

In our hands, the determination of erythrocyte cholinesterase activity by the colorimetric method of Fleisher and Pope was entirely satisfactory. It is well within the capabilities of the chemist or medical laboratory technologist. There is no necessity to refrigerate the blood samples, and because the type of anticoagulant used does not alter the results, this method should prove especially helpful to those working under field conditions.

SUMMARY

The erythrocyte cholinesterase activity was determined in two groups of cattle (30 unselected slaughterhouse cattle of various breeds, 27 purebred Aberdeen Angus cattle), using the colorimetric method of Fleisher and Pope. The average erythrocyte activity of the groups of unselected cattle was 1.26 μM (0.50 to 2.14), with a standard deviation of 0.34; of the Aberdeen Angus cattle, 1.62 μM (1.10 to 2.25), with a standard deviation of 0.27. The usefulness of this method is detailed.

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The Value of Urinalysis in Bovine Botulism

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FOR MANY YEARS, an undiagnosed disease was observed among cattle in Turkey. A research committee investigated this disease and found that it was botulism, caused by the toxin of *Clostridium botulinum* type D.

The author made the hematological examinations and urinalyses necessary for this research. The affected animals were examined in the villages, and all laboratory tests and analyses reported here were made at the time of these examinations.

Smintzis and Durin [13] examined the urine of 2 pigs with botulism. No other reports of examination of the urine of animals with botulism have been found. Therefore, it seemed that the results of our examinations should be reported.

MATERIALS AND METHODS

Examinations included observations and records of color, light-transmitting ability, odor, specific gravity, and reactions and tests for acetone bodies, indican, albumin, glucose, bile pigments, and blood. The chlorides were determined quantitatively. Robert's test for albumin, Rosenbach's modification of Gmelin's test for bile pigments, Legal's test for acetone bodies, Benedict's test for dextrose, Obermayer's test for indican, the benzidine test for blood, and Volhard and Arnold's method for chlorides were used. The most practical methods of testing had to be used, because the work was carried out under field conditions.

Urine samples from 10 supposedly normal cows from a government breeding station in the area were examined to obtain data for comparison with the findings in samples from cows with botulism.

RESULTS

The most prominent findings in the affected cattle were indicanuria, albuminuria, and glycosuria (table 2). The average amount of indican found in the urine of normal cattle is 40 to 50 mg. per liter.[9, 10, 14] The Obermayer test does not give an appreciable reaction with this amount of indican. The results of urinalyses in the 10 normal cattle are given (table 1).

DISCUSSION

Indicanuria in botulism is associated with excessive putrefaction in the alimentary tract. Of 55 affected cattle examined, all showed a full rumen and reticuloruminal atonia. Necropsies were performed on 16 affected cattle and, in each, the most prominent and constant findings were the dilation of the rumen and the presence of almost dry ingesta, especially in the rumen and omasum.

As expected, albumin was not present in the urine of the 10 normal cows tested. However, 13 of 17 affected cows showed albumin in the urine. There may be several possible causes of albuminuria in botulism. Injury of renal tissue with nephrosis was reported in bovine botulism,[11] which could cause albuminuria. Lowering of blood pressure also may cause albuminuria.[9] The arterial blood pressures were not measured in the cases reported here, but there is evidence which indicates decreased blood pressures. Animals suffering from botulism rejected water. Thirst

TABLE 1—The Data on Urinalyses in Normal Cows

Case No. and name of animal	Specific gravity	Chlorides (Gm./liter)	Indican	Albumin	Glucose
5–50 Pelin	1,037	5.12	Trace	—	—
7–47 Nuran	1,035	7.50	—	—	—
18–46 Mercan	1,032	5.80	—	—	—
18–49 Munire	1,043	6.96	Trace	—	Trace
49–40 Akkuyruk	1,040	10.44	—	—	—
56–44 Munire	1,038	7.60	Trace	—	—
60–49 Yamali	1,040	8.70	+	—	Trace
61–49 Cali	1,046	3.00	—	—	—
70–46 Esma	1,040	6.96	+	—	—
55–46 Munire	1,038	7.60	Trace	—	—

— = negative results; +, ++, and +++ = intensity of reaction.

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TABLE 2—The Data on Urinalyses in Bovine Botulism

Case No.	Course of disease	Specific gravity	Chlorides (Gm./liter)	Indican	Albumin	Glucose
12	Acute	1,026	4.64	+++	+	+
24	Subacute	1,038	3.00	++	+	−
31	Subacute	1,038	6.38	+	−	−
33	Subacute	1,036	7.60	+	−	−
36	Chronic	−	4.64	+	−	−
37	Chronic	−	7.30	+++	+	−
38	Acute	1,046	4.64	++	+	++
39	Subacute	1,043	5.80	+++	Trace	++
40	Chronic	1,020	3.48	+	+	−
41	Subacute	1,035	18.56	++	Trace	−
44	Chronic	1,020	5.56	Trace	Trace	−
46	Acute	1,052	4.64	+	Trace	+++
49	Chronic	1,048	11.60	+++	+	+
51	Subacute	1,040	6.90	+++	++	Trace
54	Chronic	1,045	9.28	+++	Trace	Trace
57	Chronic	1,026	4.06	Trace	−	−
58	Acute	1,030	14.50	++	+++	++

− = negative results; +, ++, and +++ = intensity of reaction.

causes loss of extracellular fluid and this results, in turn, in diminution of plasma volume.[2-4,7,8] The filtration pressure of kidneys and the renal blood flow is greatly diminished in dehydration.[5] Decreased filtration pressure might cause albuminuria; the lower the pressure, the easier a diffusion of albuminous substances can take place.

The loss of plasma water was reflected in the hematological findings as well. Increased erythrocyte counts and cell volumes and decreased sedimentation rates were prominent in our animals.

The quantitative determinations of chlorides were made because farmers stated that they did not give salt to animals, especially in summer months, not knowing that the salt requirement of the body is higher in hot weather than at other times. However, the sodium chloride content of the urine of affected animals proved normal, despite the fact that they were not given salt. This is not surprising, because when there is a water deficiency from inadequate intake, preservation of the body fluids requires removal of a corresponding quantity of electrolytes in the urine.

Glycosuria was found in 6 of 17 animals. In 4 of the 17, the course of disease was acute and all 4 showed glycosuria. Smintzis and Durin,[13] who made urinalyses in 2 pigs with botulism, also found glycosuria. It is difficult to find the cause of glycosuria in botulism. One possible cause may be the vascular changes of the brain, such as hemorrhage[11] and thrombosis,[12,15] since lesions in the hypothalamic tract and intracranial damage produced by apoplexy can cause glycosuria.[3,6]

Sugar is present in the urine of milking cows in which the amount of milk drops immediately. In this case, the sugar seen in urine is not glucose, but lactose. A noticeable decrease of the amount of milk in affected cows also was observed. However, glycosuria in our animals can not be attributed to this fact, because glycosuria also was seen in a male animal.

SUMMARY

Cattle (55) with botulism were examined; urinalyses were made in 17. Data from these analyses were compared with data on the urine of 10 normal cows.

The most prominent findings in botulism were indicanuria, albuminuria and glycosuria. The possible causes of the abnormalities are discussed briefly.

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Some Aspects of Glucose Metabolism in Calves After Prolonged Gestation

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GESTATION PERIODS of greater than normal length have been reported in several breeds of cattle,[3,12,13] but there have been few studies of this phenomenon. Knowledge of the factors that determine normal gestation periods and parturitions is limited. Although few cows with prolonged gestation periods, from well-managed herds of known genetic background, are available for study, we have been able to investigate one facet of this complex problem. The data reported here, plus work now under way, may help to elucidate the metabolic events which condition an animal for prolonged gestation and, conversely, those which produce normal gestation and parturition.

Prolonged gestation in cattle has been reviewed by Jasper,[12] who also recorded his observations on prolonged pregnancy in a single herd of Holstein-Friesian cows in which the condition had become a serious economic problem, ostensibly because of the breeding program being practiced in the herd. The genetic analysis of the condition by Gregory *et al.*[8] proved that the increased incidence of long, wasteful pregnancies was a result of breeding practice, and that prolonged gestation resulted when the fetal genotype was homozygous-recessive for a mutant gene that conditions long gestation periods. Gestation was normal when the fetus was heterozygous for the conditioning gene. In the series of animals studied by Jasper and Gregory, the gestation period was prolonged beyond normal (282 days) by 20 to 88 days. Most of the calves involved were females.

Prolonged gestation involves both maternal and fetal abnormalities that may be related intimately. The cow bearing the homozygous-recessive fetus is normal during the first seven and one half or eight months of pregnancy. Thereafter, the preparations for parturition are deficient. The pelvic ligaments and the vulva either do not relax or they relax incompletely, and the udder is underdeveloped. When labor begins, the uterine contractions are weak. Because of the large fetus and a dry, tight vaginal canal, delivery is impossible without assistance. The secretory activity of the mammary glands does not begin until after delivery or extraction of the fetus. Because of the difficult parturition, it is now routine practice in the Veterinary Clinic to perform cesarotomy before labor begins.

The calves are large; they continue to grow beyond the normal gestation period. All those observed by Jasper died within a few hours after delivery, and no pathological changes were found at necropsy.

Although it is recognized that deficient preparations for parturition on the part of the cow and the death of the newborn calf may not be mutually exclusive phenomena, the attack on the problem of prolonged gestation in the Hostein-Friesian breed has centered on the metabolism of the grossly normal calf. This approach was considered valid because the genotype of the fetus has a major influence on the initiation of parturition, and because a preliminary laboratory analysis indicated that the death of 1 overdue calf was associated with a hypoglycemic state. This report concerns an extension of these preliminary observations with respect to carbohydrate metabolism and other factors not described previously.

MATERIALS AND METHODS

Six calves (3 male and 3 female) were taken from Holstein-Friesian cows, overdue 35 to 62 days, from the same herd as that studied by Jasper.[12] In general, the prepartum characteristics of the cows and the gross appearances of the newly delivered calves were similar to those described previously.

Immediately after delivery, the calves were dried and held head down to clear the upper respiratory passages of mucus and fluid. The calves were then placed in a warm room, blood and urine samples were taken (the latter expressed from the bladder), and colostrum and broad-spectrum antibiotics were fed.

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Blood analyses were made as follows: Total reducing substances and fructose were determined on Somogyi filtrates of whole blood by the methods of Folin-Malmros [5] and Roe,[6] respectively. Glucose was estimated by difference. Serum sodium and potassium were determined on the Coleman flame spectrophotometer. Serum chlorides were analyzed according to the method of Schales and Schales.[17] Alkaline reserve was determined by the method of Van Slyke.[18] Urinalysis, when done, was made by these methods, adapted for urine.

The details of management of the calves differed in each case, as different methods were evaluated for prolonging the lives and improving the glycemic states of the animals. All glucose injections and infusions were made in Ringer's solution. Blood samples were taken at varying intervals during the observation periods, in order to make a prognosis on a chemical basis.

EXPERIMENTAL CALVES

Calf 1.—This animal appeared to be exceptionally strong at delivery. Attempts to prolong the life and maintain the blood sugar centered around a feeding regimen. It was fed 625 cc. of colostrum during the first eight hours and 500 cc. of milk every four hours thereafter. Because its condition deteriorated severely, it was given 200 cc. of 5 per cent glucose plus 50 mg. of hydrocortisone intravenously at 8, 30, and 69 hours of extrauterine life. It died at 92 hours of age.

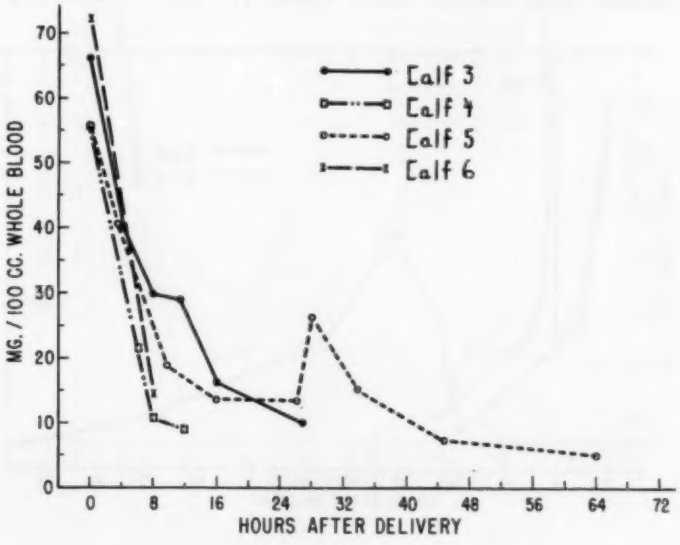
Calf 2.—This calf was weak, making no effort to move or to rise. After the initial blood sample was taken and colostrum was fed, an intravenous glusose tolerance curve was run by injecting 1.0 Gm. of glucose per kilogram of body weight. After this, the calf was infused intravenously with 5 per cent glucose and injected subcutaneously with the same fluid every five hours until death at 28 hours.

Calf 3.—No glucose tolerance curve was run on this calf. It was weak at first, but became alert and moved around the room after an initial glucose infusion, thus raising hope that it might continue to improve on glucose therapy. Consequently, the treatment given to calf 2 was repeated until this animal died at 28 hours.

Calf 4.—This calf appeared strong when delivered. After preliminary preparations, it was given an infusion of 40 units of adrenocorticotrophic hormone (ACTH) in 500 cc. of Ringer's solution, by intravenous drip in six hours. During the early stages of the infusion, the body temperature began to fall and, by the time the infusion was completed, the calf was severely depressed and shivering. Blood analyses were made and an infusion of 5 per cent glucose was given by slow intravenous drip. After nine hours of this infusion, the animal was so depressed that it was killed for necropsy.

Calf 5.—This calf was more active than any except calf 1. It was given 1 liter of 5 per cent glucose in solution by drip infusion, in 14 hours. No treatment was given for the next 12 hours, after which 1 liter of 5 per cent glucose was infused intravenously in 12 hours. At this time, the calf was showing signs of central nervous system depression but its condition did not deteriorate noticeably during the next 11 hours. A third glucose drip infusion (1 liter of fluid) was given during the third night. Because of further deterioration, the calf was killed for necropsy 67 hours after delivery.

Calf 6.—This calf was weak and its coat was drier than usual. There was evidence of fetal diarrhea, and it passed numerous watery stools while being studied. It was given a glucose drip



Graph 1—Total reducing substances in the whole blood of calves 3, 4, 5, and 6 at various intervals after delivery.

TABLE 1—Whole Blood Glucose, Fructose, and Serum Electrolytes of 6 Holstein-Friesian Calves from Prolonged Gestation Periods, Immediately After Delivery

Calf No.	Days overdue	Total reducing sugars (mg./100 cc. whole blood)	Glucose (mg./100 cc. whole blood)	Fructose (mg./100 cc. whole blood)	Serum Na (mEq./liter)	Serum K (mEq./liter)	Serum CO_2 combining power (vol./100 cc.)	Serum Cl (mEq./liter)	Packed cell volume
1	35	66	....	....	136	5.1	36	110	39
2	35	70	....	....	124	5.8	21	112	42
3	47	66	15	51	160	4.1	32	108	41
4	62	56	34	22	143	4.7	37	116	40
5	38	57	19	38	143	5.1	30	110	36
6	51	72	34	38	150	4.8	39	108	41

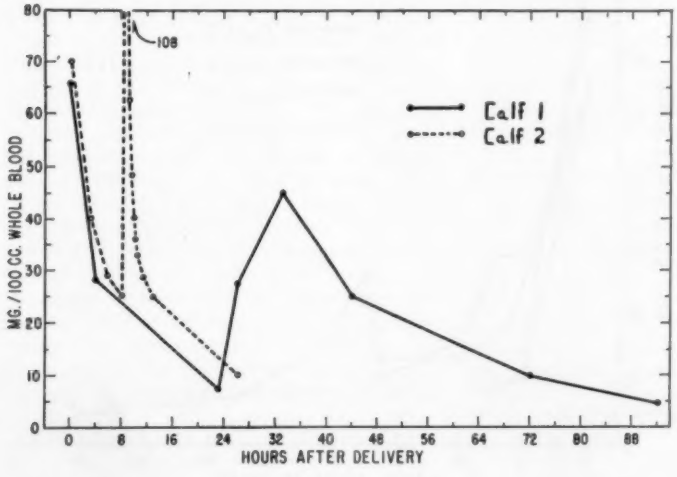
infusion; the rate of flow was difficult to control, and 1,425 cc. of 10 per cent glucose was infused in seven hours. In addition to the routine blood sampling during this period, total urine output was collected after the fetal urine had been expressed from the bladder. The urine was analyzed for glucose, fructose, sodium, potassium, and chloride ions, and compared with analyses obtained from normal newborn calves that had received identical glucose infusions. The calf was killed for necropsy when 8 hours old.

RESULTS

One preliminary analysis had indicated that terminal blood glucose levels might be low. Only after this study was started was it found that the total reducing sugars in the blood of the calf were abnormally low immediately after delivery and declined more or less steadily until death. Normal newborn calves from the same herd had initial total reducing substances of between 72 and 115 mg./100 cc. of blood. The data on the postdelivery blood samples from overdue calves (table 1) conform to those obtained from normal newborn calves from the same herd, except for the carbohydrate levels and the occurrence of acidosis in calf 2.

Despite a variety of methods of feeding and therapy, the delivery-time blood sugar levels of all 6 calves declined (graphs 1, 2, and 3). The rapid loss of sugar from the blood during the first four hours is of special interest. Of the 4 calves in which true glucose content was calculated, only in 1 was this level maintained above 10 mg./100 cc. of blood for more than eight hours. The decrease in fructose concentration was essentially normal.

During the studies on the first 5 calves, it became obvious that the concentration of blood sugar would decrease even during an intravenous infusion of glucose in Ringer's solution. Therefore, the study of calf 6 was designed to determine if the rapid loss of glucose from the vascular system was a result of renal insufficiency. For comparison, a normal term Holstein-



Graph 2—Total reducing substances in the whole blood of calves 1 and 2 at various intervals after delivery. Intravenous glucose tolerance was determined on calf 2 at 8 hours.

TABLE 2—Electrolytes and Reducing Substances in Urine of a Normal Calf and Calf 6 Before and After Infusion of 10 Per Cent Glucose in Ringer's Solution

	Chloride (mEq./liter)		Sodium (mEq./liter)		Potassium (mEq./liter)		Reducing substances (mEq./liter)	
	Normal	Overdue	Normal	Overdue	Normal	Overdue	Normal	Overdue
Initial concentration	74	35	49	56	2.9	4.2	250	660
Total amount collected during infusion	mEq.		mEq.		mEq.		Grams	
	29	48	34	31	1,920	3,420	0.753	4.63

Friesian calf, 10 hours old, was treated in the same way (table 2).

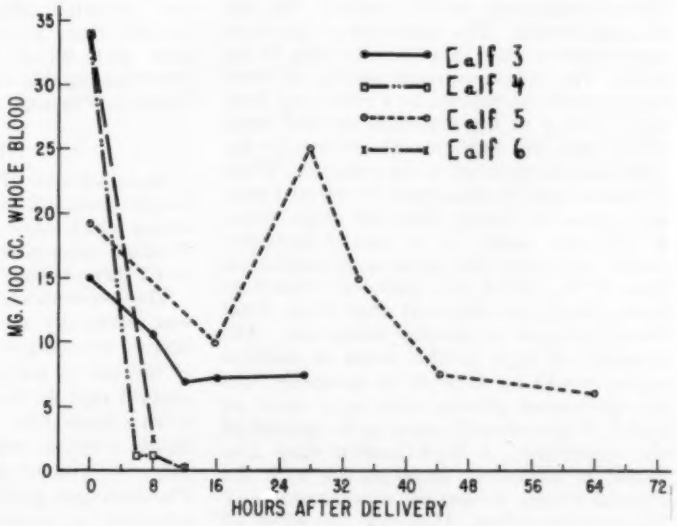
The data (table 2) also indicate an increased loss of potassium and chloride in the urine. Although the renal loss of reducing sugars was substantially greater in the overdue calf, this loss can not account for the greatest share of the blood sugar loss, because the animal was infused with a total of 71.25 Gm. of glucose during the experimental period and only 6.5 per cent was recovered in the urine.

Tolerance graphs for normal newborn calves have been made in this laboratory and the data are in agreement with those of McCandless and Dye [14] and Jarrett and Potter [11] for the young lamb. In contrast, the tolerance curve for calf 2 (graph 2) is prolonged by one and one half to two hours.

The calves in this series lived for 92, 27, 41, 28, 66, and 8 hours. Although the environmental temperature was high and all were dry and covered with cloths or heating pads, only calves 1, 2, and 5 were able to maintain rectal temperatures above 99 F. The temperature control of calves 4 and 6 was especially ineffectual; the temperatures fell to 95 F. in five hours and two hours, respectively, and remained at this level until necropsy. Since the reducing sugars of calves 1 and 2 were not fractionated, the relationship between blood glucose and temperature control is not clear.

DISCUSSION

Although there are, relatively, many reports on carbohydrate metabolism in the ungulate fetus and newborn animal, most reports have dealt with the "fructose problem," and attempts at explaining the high fetal, low maternal blood sugars.[1, 2, 4, 6, 10] There is ample evidence that the newborn calf is able to undergo long periods of starvation without a lowering of blood



Graph 3—Glucose in the whole blood of calves 3, 4, 5, and 6 at various intervals after delivery.

glucose.[7] Data on lambs and calves indicate that the level of total reducing substances, consisting of glucose, fructose and, possibly, some nonfermentable material,[4] is approximately 100 mg./100 cc. at delivery and declines within a few hours as the fructose disappears from the circulation. The newly established level of reducing substances is then maintained or is augmented if the newborn animal nurses.[11]

Comparisons with similar analyses of calves from the same herd with normal gestation periods show that the fructose level of the overdue calf was essentially normal and declined in a normal fashion. The glucose titers of the overdue calves were low and did not stabilize at any but severely hypoglycemic levels. Since fructose production is a placental function,[1,2,10] it could be postulated that this aspect of the metabolism of the aged placenta was normal. Support is lent to the idea of Alexander *et al.*[2] that fructose production by the placenta is independent of fetal glucose.

The causes of the glucose decline are obscure. Evidence from calf 6 indicated that an inefficient renal mechanism was not the sole basis for the blood glucose decline, although this calf did excrete more reducing material than did a normal calf when both were subjected to a constant intravenous infusion of glucose. Hyperinsulinemia could account for the glucose decline. The pancreas of the fetal and newborn calf is especially rich in insulin. The rapid postnatal decline in blood sugar might be related to a relatively high insulin titer in the maternal or fetal body fluids and the presence of an insulin inhibitor or antagonist in the placenta. When Passmore and Schlossmann [15] injected massive doses of insulin into the ovine fetus, it did not result in a typical hypoglycemia, although the lactic acid concentration of the blood was elevated. Furthermore, they demonstrated that ovine fetal blood contains an insulin antagonist. The presence of high insulin titers in overdue calves would be difficult to reconcile with the prolonged glucose tolerance curve of calf 2 if this should prove to be typical of the syndrome. A high insulin titer also would be ostensibly incompatible with the normal serum potassium concentration of the overdue calves. However, the effect of insulin on electrolytes of fetal and newborn calves has not been defined.

Thus far, no studies of fetal or placental glycogen have been made. The manner of handling these calves precluded the obtaining of basal levels in the tissues. Although we have no data implicating the adrenal cortex in the metabolism of the calf or in prolonging the gestation period, Hoet [9] reported that the injection of 0.5 mg. of cortisone per day to pregnant rabbits resulted in an increase in the glycogen content of the maternal and fetal placentae and an increase in the size of the fetuses; however, gestation was not prolonged. Although the placental metabolism of the rabbit may differ from that of the ruminant animal, the adrenal cortex might have some role in the increase in size of the Holstein-Friesian fetus and might explain the renal loss of potassium and chloride.

No definite correlation exists, at the present time, between the disturbed metabolism of the calf involved in a prolonged gestation period and the interplay of events that lead to normal or abnormal parturition. No one has attempted to study the interactions of the fetus, the placenta, and the maternal organism on abnormal gestation periods. The gene-conditioned gestation periods described and the abnormal metabolism of the calves may be related to the abnormal gestations in a herd of Guernsey cattle observed by Kennedy *et al.*[13] The calves were dead or lived only a few minutes after delivery and were smaller than normal fetuses. Blood glucose and blood fructose concentrations were comparable to those described for the Holstein-Friesian calves.

SUMMARY

Some aspects of carbohydrate and electrolyte metabolism have been studied in 6 calves which were delivered from Holstein-Friesian cows with gestation periods of 35 to 62 days beyond normal.

The concentration of blood electrolytes was essentially normal, but the concentration of reducing sugars in the whole blood of the calves was abnormally low and decreased rapidly during the first 4 hours of extrauterine life. Since the decrease in blood fructose was essentially normal, a defective glucose metabolism is suggested. The deranged glucose metabolism was characterized by consistent hypoglycemia, even

when the calves were fed at four-hour intervals or given intravenous infusions of glucose solutions. One calf also showed a lower glucose tolerance than did normal newborn calves.

All calves died or were killed in severe hypoglycemic states. When glucose was infused intravenously, the loss of glucose, potassium, and chloride in the urine was greater in these calves than in the normal newborn calf. The loss of glucose via the urine was not of sufficient magnitude to implicate renal mechanisms alone in the declining level of blood sugar found in the overdue calf.

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Tumors of the Bovine Acoustic Nerve—A Report of Two Cases

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TUMORS of the acoustic nerve frequently have been reported in man,[2,3,6,13,17,21,22,24] but they apparently are rare in animals. The two tumors described here and a third which has been discussed briefly by Monlux *et al.*[16] are the only acoustic nerve tumors in animals that have come to our attention. All were in cattle and all have been mentioned in earlier publications from this laboratory. Because of the infrequent occurrence of such tumors in animals, a detailed description of these two cases in cattle is presented.

The acoustic nerve tumor in man usually is described as an encapsulated oval mass of moderate size which arises in the distal part of the eighth nerve. It generally is yellow to grayish yellow and frequently is cystic. It usually engulfs the acoustic nerve and, in some cases, also envelops the facial nerve. Acoustic nerve tumors have been variously designated as neurilemmoma[13,22,24] and as neurofibroma,[2,17,21] depending on whether they were believed to originate from the cells of the sheath of Schwann or from the fibroblasts of the perineurium. According to Cushing,[2] these tumors are made up of two types of tissue; one is composed of dense interlacing bands of elongated cells, the other of loose reticular tissue. In the fibrous bands, the nuclei tend to arrange themselves in palisades and whorls. Hydropic and hyaline degeneration are not uncommon. This basic description has been accepted widely, in spite of the lack of agreement concerning the histogenesis of these neoplasms.

REVIEW OF LITERATURE

The acoustic nerve is the most common site of intracranial nerve root tumors in man,[2,6,13] but this has not been demonstrated in animals. Jungherr and Wolf[12] included two tumors of the optic nerve in an analysis of 60 cases of intracranial tumors of animals. St. Clair and Safanie[23] have described a neurofibroma of the trigeminal nerve of a dog, but their extensive search of the literature revealed only 12 intracranial tumors in over 2,000 canine neoplasms. None of these involved the acoustic nerve. Joshua and Ottaway[11] described a case of acquired hydrocephalus in a dog which was caused by tumors on the oculomotor and trigeminal nerves. The neoplasm in the bovine species described by Monlux *et al.*[16] was considered a neurilemmoma of the acoustic nerve, associated with Schwann cell tumors of other nerves. No reference to the occurrence of tumors of the acoustic nerve in animals could be found in the monographs of Feldman,[7] Guérin,[9] Jackson,[10] or Mulligan.[18]

OBSERVATIONS

The first neoplasm (126642) was from a 6-month-old Hereford calf. Clinically, the calf had shown incoordination and a tendency to circle. Examination of the brain revealed an irregular, firm, oval growth, about 1.5 inches in its greatest diameter, involving the acoustic nerve (fig. 1). The surface of the tumor was hemorrhagic and the deeper portions were grayish. The second tumor (D-1222) was found in an 18-month-old steer. The clinical history stated that the animal held its head to one side and circled to the left. The animal leaned against objects and would walk along fences, apparently in an attempt to support itself. Necropsy revealed a fibrous tumor in the cerebellopontine angle, which measured 2 by 1 by ¾ inch (fig. 2). Both of these tumors were encapsulated, and nerve fibers were visible on the surfaces of both.

Microscopically, the first specimen (126642) was composed of a sparse stroma of interlacing strands of elongated neoplastic cells, which enclosed areas of loose reticular tissue (fig. 3). Reticular tissue predominated in this tumor, and the entire mass was cystic and edematous. Considerable hematogenous pigment was present in some areas. Nuclear palisading was not observed.

Longitudinal sectioning of the second specimen (D-1222) in the dorsoventral plane revealed the presence of a large nerve trunk included in the most dependent part of the mass (fig. 4). A large nuclear group was seen medial to the neoplasm and ventral to the flocculus. This

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was thought to be the ventral cochlear nucleus of the acoustic nerve. Fibers of the acoustic nerve could be traced into this nucleus. The tumor was well demarcated and was exerting sufficient pressure on the brain stem to cause considerable distortion of the fiber tracts. Numerous long individual strands, morphologically similar to the fibers of the acoustic nerve, were present in the tumor (fig. 5). These structures had a haphazard arrangement, and probably represented the "neurites" spoken of by Stout [24] as being found in expansive growths which are invading large nerve trunks. They appeared to be fibers of the nerve which had been engulfed by the neoplasm. No distinct cysts were seen in this tumor, although a small amount of perivascular edema was present. Definite palisading of the nuclei was not recognized.

The essential cell of both tumors was characteristic of those described for the acoustic nerve tumor in man, *i.e.*, an elongated cell forming a syncytium and having a moderate-sized fusiform nucleus (fig 6). Special stains showed that these cells were capable of producing reticulin and collagen. The blood supply of both tumors was adequate and no areas of necrosis were recognized. The walls of many of the vessels were thickened and contained dense hyaline material.

Discussion

The wide divergence of opinion as to the histogenesis of tumors of the acoustic nerve is illustrated by the number of specific names that have been applied to them. Cushing [2] listed more than 20 synonyms which have been used to identify these tumors. The most widely separated views are those of Masson [14] and Stout,[24] who classified them as schwannomas or neurilemmomas, and of Rhoades and Van Wagenen,[21] who regarded them as perineural fibroblastomas. In spite of this difference of opinion, there seems to be general agreement that: (1) These tumors arise from nerve sheaths; (2) they are made up of two types of tissue—dense interlacing bands of elongated cells and a loose reticular tissue; and (3) the essential cell of the neoplasm is capable of producing collagen and reticulin.

Gore [8] has reported the remarkable similarity of the morphological and staining characteristics of the Schwann cell and the

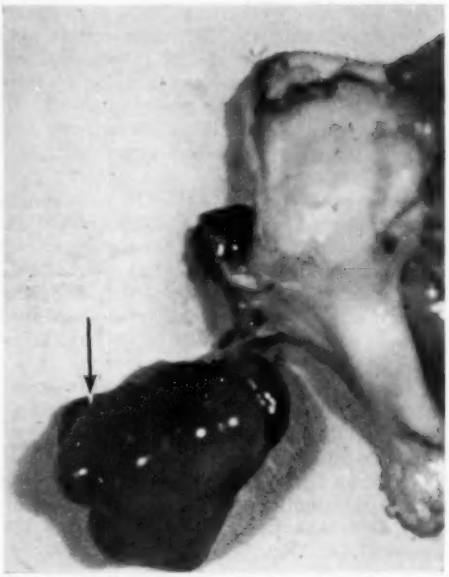


Fig. 1—Tumor of the bovine acoustic nerve (126642). The tumor (arrow) is an oval mass, about 1.5 inches in greatest diameter, which is affecting the acoustic nerve. The surface of the neoplasm is hemorrhagic.

Fig. 2—Tumor of the bovine acoustic nerve (D-1222). The mass of the tumor involves the eighth nerve, but the facial nerve (arrow) also is being engulfed.

fibroblast. Moreover, tissue culture studies[19] have shown that the Schwann cell is able to produce collagen. All of these facts emphasize the importance of Dublin's statement[5] that "this close resemblance of cell types has provoked a long, noteworthy and rather pointless controversy over histogenesis."

Tumors of the nerve sheath of animals have received relatively little attention. Monlux and Davis[16] considered the Schwann cell the basic element of neoplasms of the peripheral nerves in cattle. All but one of their tumors were considered benign and, when multiple, they were thought to be of multicentric origin. They indicated that the absence of skin lesions in their material suggests that neurofibromatosis (Recklinghausen's disease) is peculiar to man. Drieux and Mendoza[4] and Peyron and Drieux[20] have designated a neoplastic condition in cattle, in which they felt that the Schwann cell was the essential element, as neurofibromatosis.

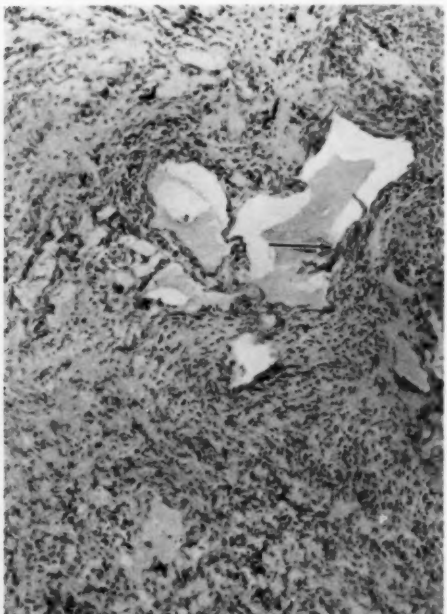
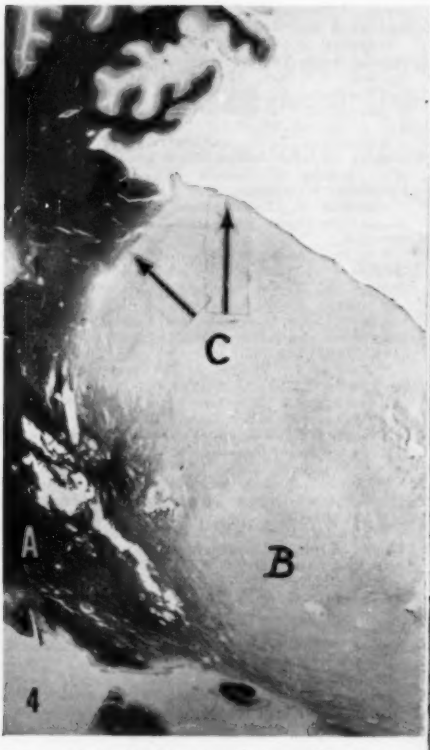


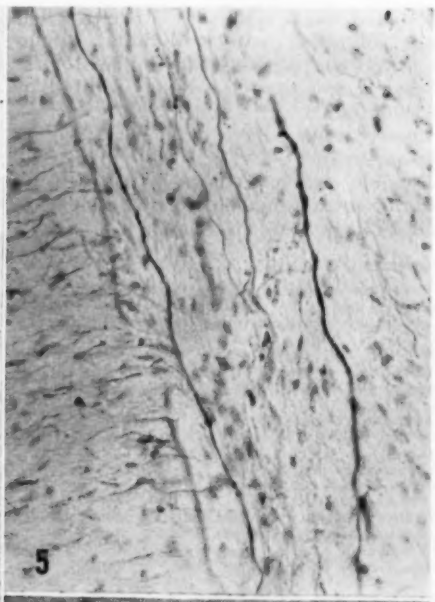
Fig. 3—Tumor of the bovine acoustic nerve (126642). The cystic and edematous character of this neoplasm is demonstrated. There is a relatively sparse stroma composed of strands of elongated cells (arrow), but most of the cells in this field have a loose reticular arrangement with no distinct polarity. H & E stain; x 110.

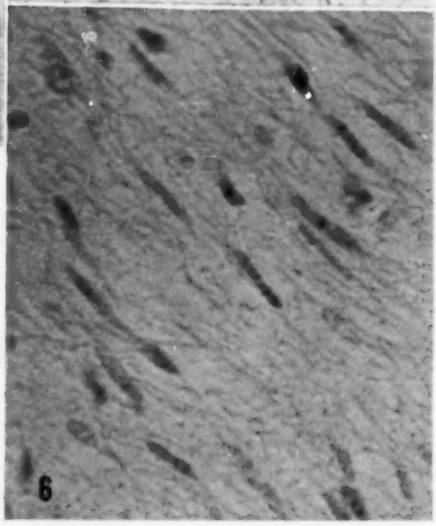
Moreover, they did this in the absence of cutaneous manifestations, which tends to increase the already great confusion over the nomenclature of these tumors. Multiple skin lesions resembling neurofibromatosis in man are rare in animals, having been described only in the chicken[10] and in the horse.[1]

The bovine acoustic nerve tumors being described apparently were solitary lesions which were histologically benign and well encapsulated. They contained two types of tissue—a dense fibrous tissue made up of a syncytium of elongated cells and a loose reticular tissue. We believe that they are related to the Schwann cell tumors in the bovine species described by Monlux and Davis[16] and should be classified as schwannomas.

The rarity with which these neoplasms are found in animals presents the opportunity for some interesting speculations. Cushing[2,3] stated that the acoustic nerve is the most common site of intracranial tumors of nerve roots in man and cited only three instances of involvement of other cranial nerves. Kernohan and Sayre[13] commented on the rarity of such tumors in situations other than on the eighth nerve. Tumors of the trigeminal[11,23] and the oculomotor[11] nerves of dogs have been described previously. Thus, the recorded number of acoustic nerve tumors in animals is approximately equal to the number of reported tumors of other cranial nerves, if the optic nerves are excluded from consideration. It is doubtful that these citations represent the true picture. Acoustic nerve tumors probably produce noticeable clinical signs in animals only after they have reached sufficient size to cause distortion of the cerebrospinal axis, and when these signs do develop they may be attributed to other causes. In cattle, such signs frequently are associated with listeriosis, and this was the clinical diagnosis in both of the cases being discussed. In many instances, examination of the brain is not included as part of the routine necropsy in animals unless the signs have been such as to make it mandatory. This is particularly unfortunate, as it applies to intracranial nerve root tumors which are sometimes discovered as an incidental necropsy finding in man. It is possible that many cases have been overlooked in animals and that these tumors are not as rare as the small number of reports might suggest.







Tumor of the bovine acoustic nerve(D-1222)

Fig. 4—A large nerve trunk (A) is visible below the lighter-colored mass of the growth (B). The tumor capsule (C) is evident. Galantha stain; x 3.

Fig. 5—Individual nerve fibers are shown. These strands were seen scattered throughout the tumor and appeared to be fibers of the acoustic nerve which have been engulfed by the neoplasm. Galantha stain; x 220.

Fig. 6—The elongated, syncytial cells have a distinctly polar arrangement. H & E stain; x 630.

An estimate of the incidence of intracranial tumors in animals will not be feasible until examination of the brain becomes routine necropsy procedure.

SUMMARY

Two tumors of the acoustic nerve in cattle, both of which showed many of the features of the acoustic nerve tumor in man, have been described. These neoplasms were situated within the cranium on the root of the eighth nerve, and were composed of the two types of tissue commonly found in the lesion in man. The essential neoplastic cell was an elongated syncytial cell which was arranged in interlacing bands. One of the tumors was composed primarily of loose, reticular tissue, whereas

the second was a more solid structure. The latter contained many individual, apolar structures that appeared to be fibers of the acoustic nerve that had been engulfed by the neoplasm.

These tumors have been classified as schwannomas.

Possible reasons for the paucity of reports of intracranial nerve root tumors in animals have been considered.

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Neural Mechanisms in Sexual Behavior
II. Gross Neuroanatomical and Correlative Neurophysiological Studies of the External Genitalia of the Bull and the Ram

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DURING PREVIOUS STUDIES,[11,15,16] it became apparent that detailed descriptions, including illustrations, of the peripheral nerve supply to the external genitalia of the bull were not available. Textbooks of veterinary anatomy,[2,4,19,20,23] laboratory guides,[5,8,10,21] and other reports[15,16,24] generally describe the intrapelvic course of the sacral spinal nerves but fail to elaborate on the peripheral distribution of many of their terminal branches. Neither do they describe the terminal branches of the inguinal, lumbar, and thoracic nerves as they relate to the external genitalia.

Few studies have been made in large domestic animals which combine gross anatomical dissections with neurophysiological investigations. By combining these techniques, much useful information can be secured and some errors inherent in each method can be avoided. The purpose of this paper is to present the results of gross anatomical and correlative neurophysiological observations on the nerve supply to the external genitalia of the bull and the ram.

MATERIALS AND METHODS

Anatomical Studies, Bulls.—Dissections were made on parts from 25 animals: (1) In 7 bulls (5 mature and 2 immature), the inguinal and preputial areas were dissected in detail; (2) in 1 mature bull, the sacral, inguinal, and preputial areas were dissected; (3) 8 bulls were observed as dissection progressed in the student gross anatomy laboratory; (4) in 2 adult bulls, the sacral plexus was dissected at necropsy; (5) the lateral thoracic nerve and its branches were dissected at necropsy in 2 young bulls; (6) the dorsal nerves, arteries, and veins of the penises of 7 adult bulls were studied by dissecting when they were removed at slaughter. All animals listed under the first three items were embalmed specimens; the remainder were dissected in the fresh state.

To record the findings, sketches were made or photographs taken as the dissections progressed. Using the information thus obtained, as well as that reported earlier,[15,16] illustrations were prepared showing in detail the nerve supply to the external genitalia in the bull (fig. 1–4).

The description included in this report represents the most consistent findings. The variations from these will be pointed out at the appropriate times. Wherever applicable, the Nomina Anatomica[8] terminology will follow, parenthetically, each anglicized anatomical term as it is introduced.

Anatomical Studies, Rams.—Dissections were made of the sacral areas of 17 sheep. In 15 of these, the observations were made at the time of surgery, prior to electrophysiological studies, and at necropsy. In 2 embalmed specimens, the sacral areas were dissected in detail. Only major anatomical differences between the ram and the bull will be cited.

Neurological Studies.—A total of 5 calves and 15 rams were used in this portion of the study. The general method and equipment used have been described in detail previously (fig. 5).[11] Anesthesia was accomplished with diallylbarbituric acid and urethane (Dial-Urethane*) administered intravenously. Tracheotomy was performed to prevent inhalation of saliva or regurgitated ingesta.

Laminectomies were performed in the midthoracic regions to permit transection of the spinal cord, and in the lower lumbar and sacral regions to expose the dorsal rootlets of the sacral segments. The exposed areas of the spinal cord were covered with warm (38 to 40 C.) mineral oil. The peripheral nerves were surgically isolated, transected, and covered with warm mineral oil.

Silver wire electrodes were used for both stimulating and recording. Supramaximal, single-shock

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* The Dial-Urethane was supplied by Ciba Pharmaceutical Products Inc., Summit, N.J.

stimuli were delivered to the peripheral nerves, using a multichannel thyratron stimulator or a square wave stimulator.

The compound action potentials resulting in the dorsal roots from stimulation of the central portions of transected peripheral nerves were amplified by means of condenser-coupled, differential amplifiers with long time constants, and recorded and photographed on the face of a sweep synchronized cathode-ray oscilloscope by a continuous recording camera.

Electromyographic recordings were made in a similar way, except that bipolar steel electrodes were inserted, 5 mm. apart, in the muscles under consideration. In these instances, electrical stimuli were delivered to the distal portions of transected peripheral nerves.

OBSERVATIONS, RESULTS, AND DISCUSSION

Central Origin of Nerves to the External Genitalia.—The external genitalia receive their nerve supply by three routes, *i.e.*, cranial, middle, and caudal. The peripheral nerves comprising the cranial route are the lateral thoracic (n. thoracolateralis) which originates from the ventral branches (ramus ventralis) of the eighth cervical (C8) and first and second thoracic spinal nerves (T1 and T2) and the ventromedial branches of the ninth to thirteenth thoracic (T9 to T13) and first and second lumbar (L1 and L2) spinal nerves (fig. 3). The peripheral nerves following the middle route are the inguinal nerves (n. genitofemoralis) which originate from the ventral branches of the second, third, and fourth lumbar spinal nerves (L2 to L4). The peripheral nerves comprising the caudal route are numerous and originate from the ventral branches of all five sacral spinal nerves (S1 to S5). Electrophysiological evidence which supports these gross

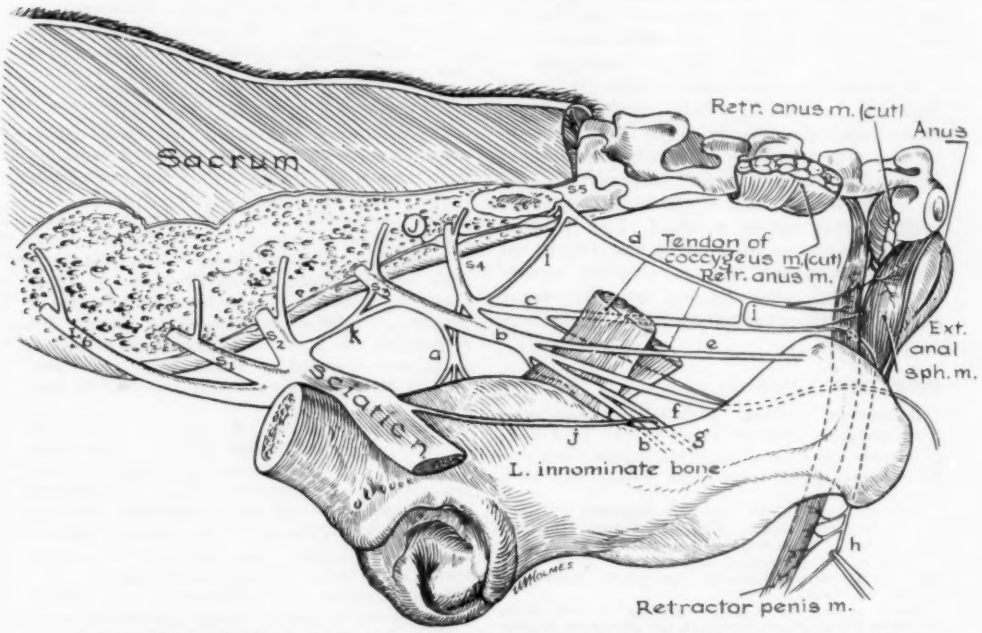


Fig. 1—Schematic line drawing of the left sacral plexus in the adult bull, representing what may be considered the typical arrangement of the nerves derived from the first through fifth sacral segments. Notice that the S2 contribution (k) to the pudendal nerve (b) is small S4 continues caudally as the middle hemorrhoidal nerve (c). It passes through the substance of the retractor penis muscle and continues ventrally to ramify in the retractor penis muscle. The caudal hemorrhoidal nerve (d) has two anastomoses (i) with the middle hemorrhoidal nerve. The pelvic nerve (a) is derived from S3 and S4. The proximal and distal cutaneous branches (e, f) of the pudendal nerve come off this nerve separately and just cranial to the anterior border of the coccygeus muscle. The last pelvic branch from the pudendal nerve (b), the deep perineal nerve (g), is given off just caudal to the caudal border of the coccygeus and retractor anus muscles. The anastomosis between the sciatic and the deep perineal nerves is illustrated (j).

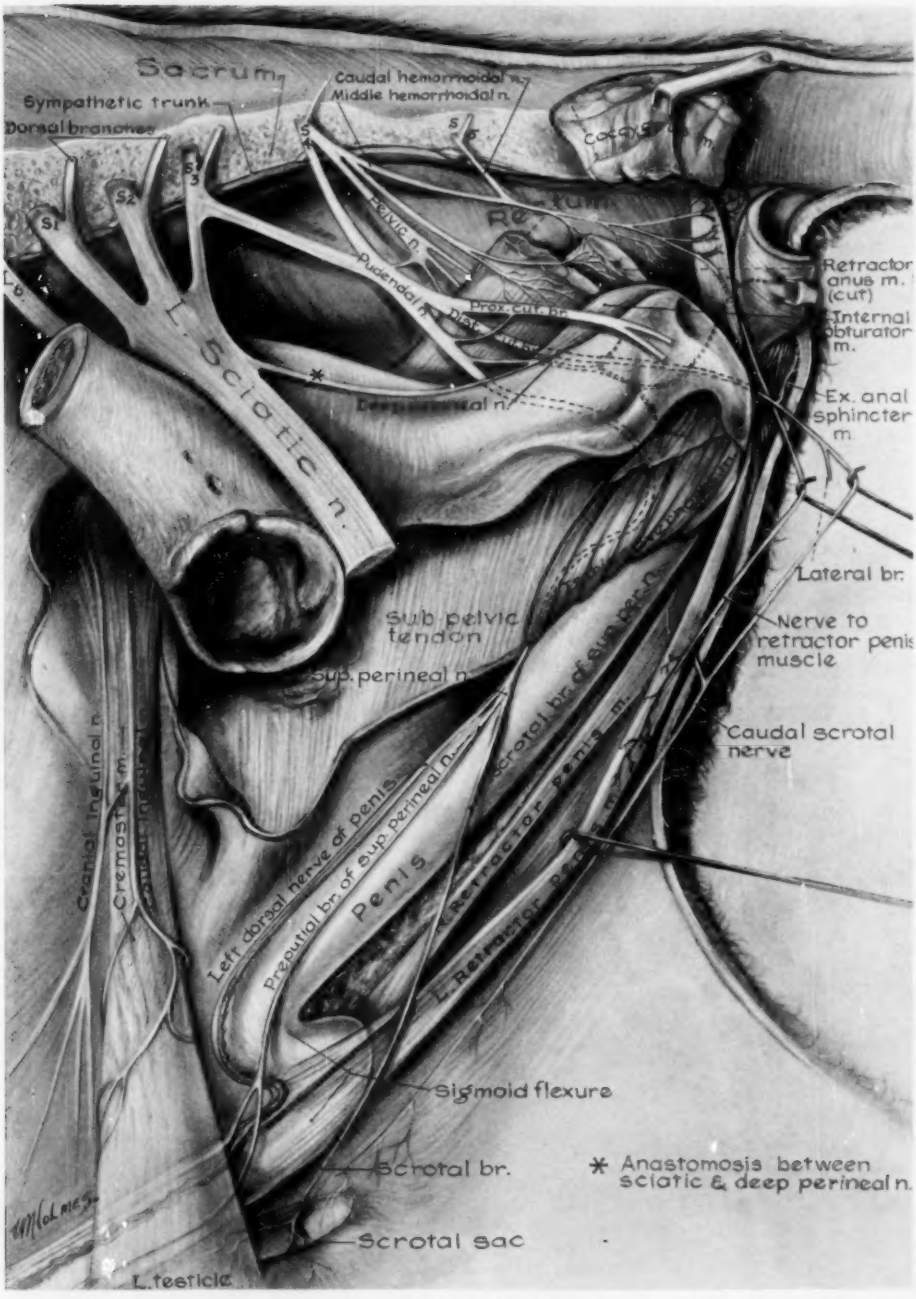


Fig. 2—The nerve supply to the proximal parts of the external genitalia in the bull.

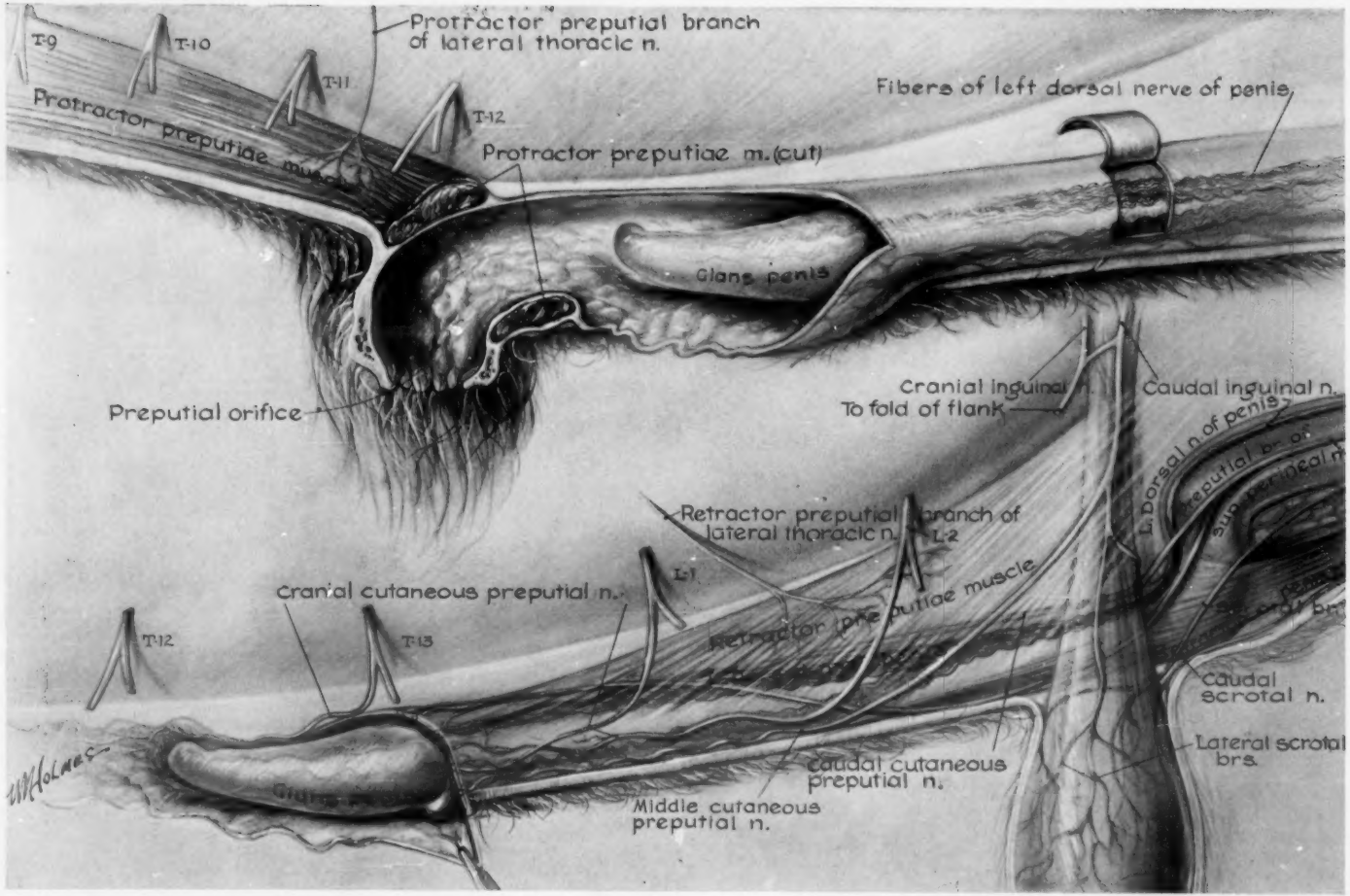

T-9
T-10
T-11
T-12
Protractor preputial branch of lateral thoracic n.
Protractor preputiae muscle
Protractor preputiae m.(cut)
Fibers of left dorsal nerve of penis
Glans penis
Preputial orifice
Cranial inguinal n.
To fold of flank
Caudal inguinal n.
L. Dorsal n of penis
Retractor preputial branch of lateral thoracic n.
L-2
L-1
Cranial cutaneous preputial n.
T-12
T-13
Caudal scrotal n.
Lateral scrotal brs.
Caudal cutaneous preputial n.
Middle cutaneous preputial n.

Figure 3

anatomical observations as to the central origin of these nerves will be presented when the peripheral distribution of these nerves is discussed.

The autonomic nerve supply to the external genitalia has not been determined fully, particularly concerning correlative electrophysiological determinations. The findings, as a result of the gross dissections conducted in this study, are consistent with those of St. Clair.[24]

The sympathetic trunks lie along the sides of the bodies of the vertebrae in the lower thoracic regions and become more ventrally located in the lumbar and sacral areas (fig. 1, 2). The trunks may anastomose in the sacral area but, in this study, the findings indicate that the trunks join more commonly at the second or third coccygeal vertebrae and the gray rami continue into the tail from that point as a single minute nerve. A sympathetic ganglion (lateral or chain ganglion) usually is located on the sympathetic trunk corresponding to each spinal nerve (fig. 1, 2). Rami communicantes, up to four, connect the sympathetic fibers to the sympathetic trunk where they may synapse with neurons located in the lateral ganglia of the sympathetic trunk. These neurons give rise to postganglionic fibers which return to the spinal nerves, via gray rami communicantes, for distribution to the body wall. Of particular interest are the gray rami going to S2, S3, and S4. These sympathetic fibers eventually supply the retractor penis muscle.

The parasympathetic nerve supply to the external genitalia arises from the sacral segments of the spinal cord and are carried in the ventral roots of sacral spinal segments 2, 3, and 4. They continue for a short distance in the respective spinal nerves and then apparently separate from them to help form the pelvic nerve (n. splanchnicus pelvinus) (fig. 1, 2). This nerve enters into the formation of the pelvic plexus (plexus pelvinus) in the pelvic cavity. The parasympathetic fibers apparently are distributed, as in other species, via the pelvic nerve and pelvic plexus to all the pelvic viscera and genital organs except, perhaps, the testes.[9, 13]

Caudal Route of Nerves Supplying the External Genitalia.—The caudal route includes the ventral branches of the sacral spinal nerves which form the sacral plexus (plexus sacralis). The most typical arrangement of this plexus is illustrated (fig. 1). From this plexus, three nerves arise which supply the genitalia. These are the pudendal and the middle hemorrhoidal nerves, and the anastomotic branch between the sciatic and the deep perineal nerves. Of these, the pudendal nerve is the largest. It was observed to arise, with two exceptions, from the ventral branches of S2, S3, and S4 (fig. 1). Two specimens did not have a contribution to the pudendal nerve from S2 (fig. 2). The fourth sacral segment contributed to the pudendal nerve in all but one instance. In addition to being the most consistent contributor to the pudendal nerve, S3 also regularly contributes the largest portion.

The central connections of the afferent fibers in the pudendal nerve were determined electrophysiologically by stimulating the pudendal nerve just distal to its origin from the sacral plexus and recording the compound action potential, if any, from the dorsal rootlets of segments of the spinal cord. The results of this procedure in 1 animal are illustrated (fig. 6). In all animals, action potentials were obtained from the dorsal rootlets of S3. In the majority of experiments, S2 and S4 also were involved. In a few, potentials were found in S5. No differences between calves and sheep were observed.

At its origin, the pudendal nerve lies deep and medial to the sacrosciatic ligament and in close apposition to it. The nerve courses in a caudoventral direction to the region of the cranial part of the lesser sciatic foramen (fig. 1, 2). In several instances, muscle branches to the coccygeus and retractor anus muscles were observed to arise from the pudendal nerve in the proximal third of the portion between its origin and the lesser sciatic foramen. It was more common, however, to find these muscle branches arising from the distal third of the segment of the pudendal nerve (fig. 1) or from the middle hemorrhoidal nerve (fig. 1, 2), or both.

Legend for Illustration on Opposite Page

Fig. 3—The nerve supply to the distal parts of the external genitalia in the bull.

In most specimens, the pelvic nerve was formed from the pudendal nerve or the sacral contributions to it (fig. 1). The branches which form the pelvic nerve arise from the middle third of the pudendal nerve. In only one instance did the major part of the pelvic nerve originate from the ventral branch of S4. In all experiments on the pelvic nerve, stimulation of it resulted in the appearance of action potentials in the dorsal rootlets of S2, S3, and S4 (fig. 7).

At the lesser sciatic foramen, the pudendal nerve passes lateral to the coccygeus muscle and two rather large branches arise from its dorsal aspect. The first of these is the proximal cutaneous branch and the second is the distal cutaneous branch of the pudendal nerve (fig. 1, 2, 8).[7] In the ram, only one cutaneous branch is present. The deep perineal[16] nerve leaves the pudendal nerve just distal to the origins of the cutaneous branches (fig. 1, 2). The pudendal nerve then passes in a caudomedio-ventral direction. It lies on the internal obturator muscle and, at the midline, the nerve reaches the ischial arch and, with its fellow, passes over the arch, deep to the penis (fig. 2). After rounding the ischial arch, the pudendal nerve becomes embedded in the substance of the subpelvic tendon for a short distance. Upon emerging from the subpelvic tendon, it terminates shortly by dividing into the dorsal nerve of the penis and the superficial perineal nerve.[16]

In the bull, the first major branch of the pudendal nerve, the proximal cutaneous branch (fig. 1, 2), arises from the pudendal nerve just cranial to the cranial border of the coccygeus muscle. The proximal and distal branches may arise by a common trunk (fig. 2). The proximal cutaneous branch runs in the substance of the sacrosciatic ligament to emerge from it at its caudal rounded edge, the sacrotuberous ligament. The nerve then becomes subcutaneous and divides into medial and lateral branches, which are distributed to the skin over the semitendinosus muscle.

Electrophysiological studies show that, in most of the animals, the afferent fibers in the proximal cutaneous branch enter through the dorsal rootlets of S3 and S4. In a few animals, they also enter through S2 and S5. Stimulation of the distal cut end of this nerve did not result in any contraction of striated muscle. On the basis of these results, it can be considered entirely a cutaneous nerve.

The distal cutaneous branch of the pudendal nerve arises just distal to the proximal cutaneous branch. Generally, this is at the point where the pudendal nerve passes lateral to the coccygeus muscle in the lesser sciatic foramen (fig. 1, 2, 8). This branch courses medial to the dorsal process of the tuber ischium. The nerve emerges in the ischiorectal fossa and, at the level of the ischial arch, divides into a small lateral and a larger medial branch. The lateral branch is distributed to the skin over the semimembranosus muscle. The medial component is joined at the midline by its fellow from the opposite side to form the caudal scrotal nerve (fig. 2).[16] It continues along the midline, where some cutaneous branches are given off, then ramifies in the caudal scrotal wall (fig. 2). The central origin of the distal cutaneous branch of the pudendal nerve, as determined electrophysiologically, is the same as the proximal branch. On the basis of its gross distribution and the results of stimulation of the distal cut end without obtaining muscular contractions, it also can be considered purely a cutaneous nerve.

The deep perineal nerve is given off from the pudendal nerve just distal to the caudal border of the lesser sciatic foramen (fig. 1, 2). It is joined by an anastomotic branch from the sciatic nerve. The deep perineal nerve quickly breaks up into several branches which are distributed to the muscle derivatives of the cloacal sphincter of the embryo,[6, 14] *i.e.*, the bulbocavernosus and ischiocavernosus muscles, as well as the external anal sphincter and urethral muscles. The terminal rectum also receives branches from this nerve.

A nerve branch from the sciatic (anastomosis between sciatic and deep perineal nerve; fig. 2), which passed lateral to the sacrosciatic ligament and entered the lesser sciatic foramen to become associated with or actually joined with the deep perineal nerve, was present in all dissections (fig. 9). Branches from this anastomotic branch were often found going directly to these muscles. The deep perineal nerve and the anastomotic branch between it and the sciatic will be discussed together, as they have been found to contribute to the nerve supply of the same muscles. There is considerable controversy regarding the name, function, and distribution of the anastomotic

branch. Because of its unique arrangement and the duality of the nerve supply to the muscles of the cloacal sphincter, these nerves are being investigated in considerable detail and will be the subject of a separate report.[12] Only the gross anatomical observations will be discussed here.

Reimers[22] apparently has called this nerve the caudal cutaneous femoral nerve. He describes it as passing lateral to the pudendal nerve and ramifying in the skin over the so-called "buttock muscles." Martin and Schauder[19] refer to this anastomotic branch as a branch of the pudendal nerve. Habel[8] has described the anastomotic branch as joining the pudendal nerve. That the anastomosis from the sciatic joins the deep perineal and not the pudendal nerve is shown (fig. 9).

The dorsal nerve of the penis is formed when the pudendal nerve terminates by dividing into it and the superficial perineal nerve shortly after rounding the ischial arch. The dorsal nerve of the penis (fig. 4) becomes intimately associated with the dorsolateral aspect of the penis. As the nerves round the ischial arch, they lie medial to the dorsal arteries of the penis. This relationship becomes reversed in the middle third of the segment of the penis between the ischiocavernosus muscle and the first bend of the sigmoid flexure (fig. 4A). At the second bend of the sigmoid flexure, the dorsal nerves of the penis again become located medial to the arteries, due to the ventrolateral course the arteries take from this point on.

The nerves are slightly wavy in nature proximally and become progressively more so distally (fig. 4A, 4B). Small branches

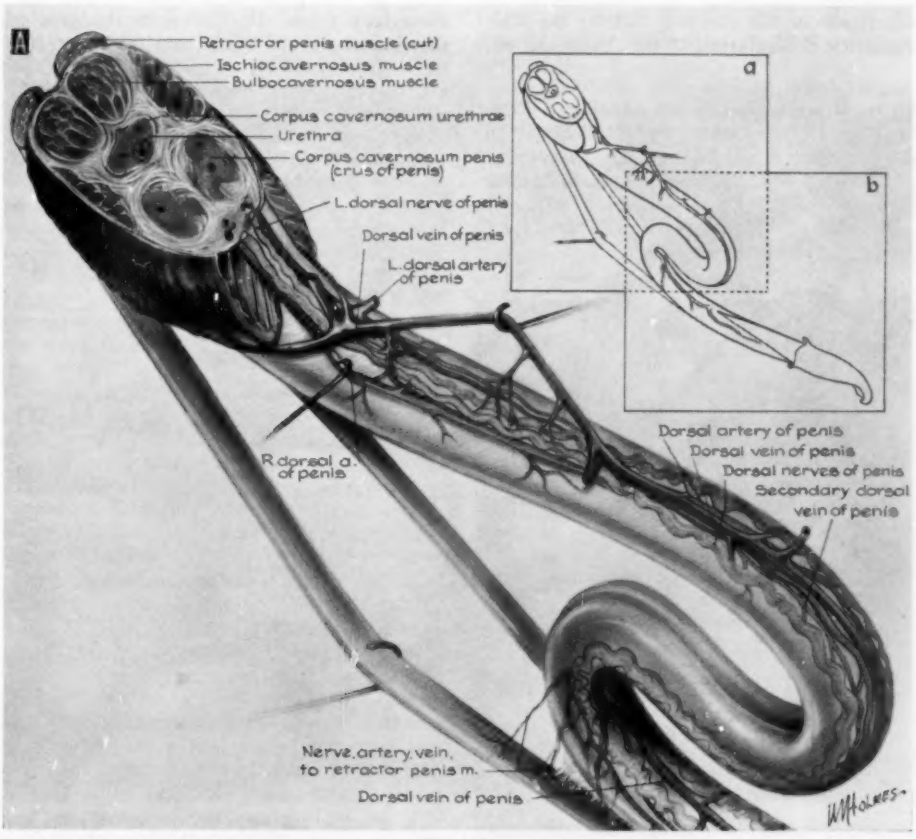


Fig. 4A—Relationship of the dorsal nerves, arteries, and veins of the bull's penis (proximal part).

which perforate the tunica albuginea of the penis are given off throughout the length of the nerve; however, the greatest number of terminal branches are found in the area of the glans penis. About halfway between the second bend of the sigmoid flexure and the fornix of the prepuce, the dorsal nerve breaks up into two or three secondary branches. These pass under the dorsal fibrous tunic and are distributed in a pattern of terminal branches, as illustrated (fig. 4C). In this area, the nerves are found mainly on the dorsal and lateral aspects and the blood vessels take a ventral position. Fine but grossly dissectable nerve branches from the dorsal nerve of the penis were observed to be distributed to the retractor penis muscle (fig. 4A, B).

Stimulation of the central cut end of the dorsal nerve of the penis resulted in action potentials in S2 and S3, but in no other segments. Stimulation of the distal cut end of the dorsal nerve of the penis did not produce any contractions of striated muscle, but did result in contraction of the retractor penis muscle. Studies of reflexes involving the retractor penis muscle, a smooth muscle, will be reported elsewhere.[17] From the results of the stimulation experiments, it appears that the dorsal nerve of the penis is sensory to the penis and contains sympathetic fibers to the retractor penis muscle.

The dorsal arteries of the penis, as mentioned earlier, course ventrolaterally distal to the second bend of the sigmoid flexure. In the area of the glans penis, they are almost entirely ventral in position (fig. 4B, 4C).

The dorsal vein of the penis usually is single and follows the middorsal aspect of this organ. It has many branches to the body of the penis and anastomoses with secondary veins. It divides in the area of the dorsal fibrous tunic, and the terminal

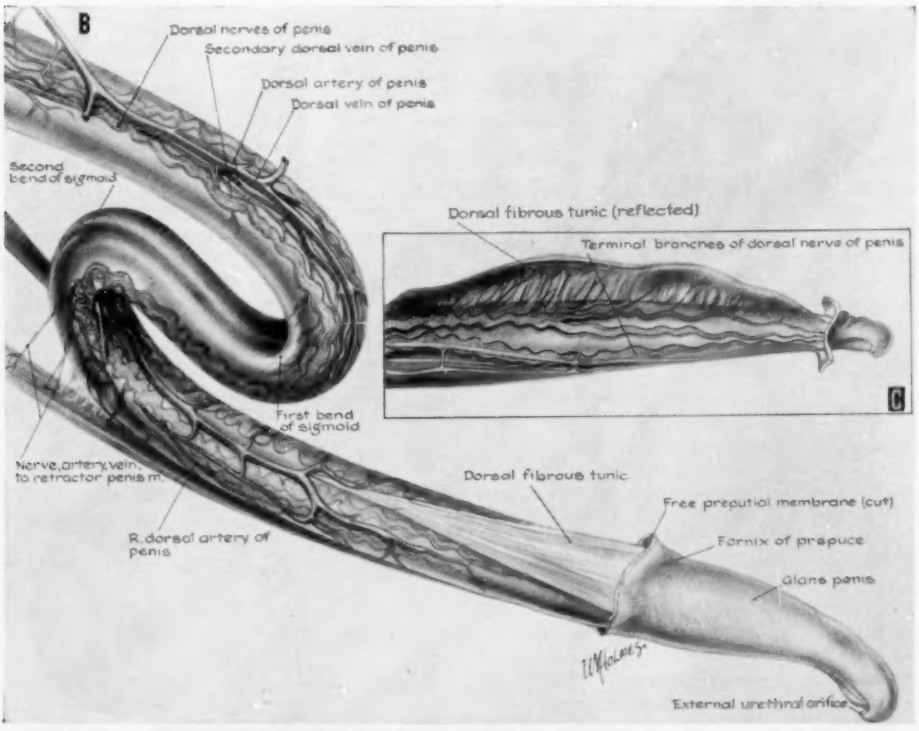


Fig. 4B and 4C—Relationship of the dorsal nerves, arteries, and veins of the bull's penis (distal part).

branches pass laterally and ventrally to be distributed over the ventrolateral aspect of the glans and the area just caudal to it (fig. 4B, 4C).

The dorsal fibrous tunic, found in all specimens dissected, has a variable proximal attachment, but diverges distally to envelop almost the entire glans. Fibrous bands may attach the tunic to the penis proper at various points but, generally, it is freely reflectable with only areolar tissue between it and tunica albuginea of the penis (fig. 4B, 4C).

The superficial perineal nerve,[16] the other terminal branch of the pudendal nerve, apparently is the homologue of the nerve which, in the cow, is called the perineal nerve by St. Clair [24] and the mammary branch of the pudendal by Habel.[7] This nerve usually gives rise to two branches which could best be termed scrotal and preputial branches of the superficial perineal nerve in the bull (fig. 2) and mammary branches of the pudendal nerve in the cow. In some dissections, the scrotal and preputial branches arose independently as terminal branches of the pudendal nerve. The scrotal branch of the superficial perineal nerve proceeds ventrally to the scrotum and gives rise to branches which terminate in the cranial aspect and medial septum of the corresponding half of the scrotum (fig. 2, 3). The preputial branch of the superficial perineal nerve passes cranially, may penetrate the retractor preputiae muscle, and divides into several branches, usually three or four, which then course along the penis and free part of the preputial membrane. Some branches are given off to the preputial membrane. The nerves continue as far cranially as the preputial orifice, where they terminate in cutaneous branches (fig. 3). These will be termed, collectively, caudal preputial cutaneous nerves.

The superficial perineal nerve is not a discrete entity in the ram as it is in the bull. It seems to consist of fine cutaneous rami from the dorsal nerve of the penis.

The central connection of the superficial perineal nerve was determined electrophysiologically in a single animal. In this animal, the compound action potential was recorded from the dorsal root of S2. Stimulation of the distal segment of this nerve did not produce any contraction of striated muscle. These results, plus its distribution, suggest that it is principally a sensory nerve to the scrotum and the prepuce.

The middle hemorrhoidal nerve, which is formed mainly from the ventral branch of the fourth sacral nerve, may anastomose with the pudendal or the caudal hemorrhoidal nerve, or both (fig. 1, 2). It lies medial to the coccygeus muscle as it runs caudally. The nerve may give off branches to the coccygeus and retractor anus muscles. After giving branches to the skin around the anus, the terminal part of the nerve passes through the substance of the retractor penis muscle (fig. 1, 2). The nerve then turns ventrally and ramifies in the retractor penis muscle (fig. 2). Habel [7] refers to the terminal part of the middle hemorrhoidal nerve in the cow as the perineal nerve. It would appear unnecessary to give this terminal part a separate name. Also, it results in confusion with the superficial perineal branch, one of the two terminal branches of the pudendal nerve.

In the ram, the middle hemorrhoidal nerve terminates in the proximal part of the retractor penis muscle. Stimulation of the central cut end of the middle hemorrhoidal nerve resulted in the appearance of action potentials in the dorsal rootlets of S3 and S4 in all instances. In about one half of the experiments, potentials were

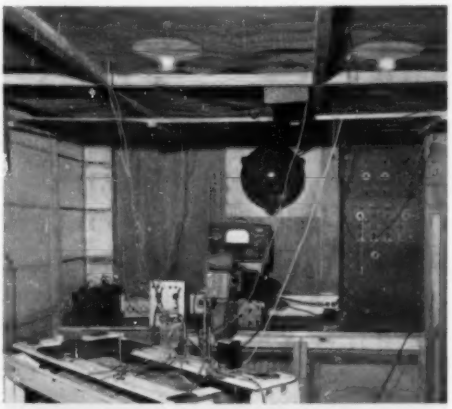


Fig. 5—Electophysiological equipment used: animal holder with electrodes in position (foreground), two differential amplifiers (rear left), cathode-ray oscilloscope with continuous record camera attached, audiofrequency generator resting on oscilloscope (rear center), and multichannel stimulator (rear right). The recording room is surrounded by wire screen to reduce electrical interference.

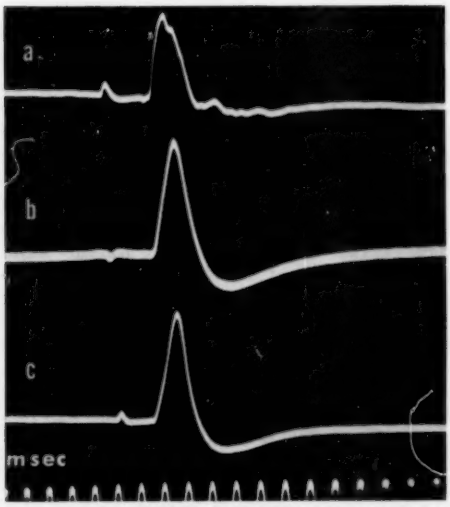


Fig. 6—Action potentials recorded from the dorsal roots of (a) S2, (b) S3, and (c) S4 after stimulation of the pundenal nerve in a ram.

recorded in S2 and, in the other half, in S5. The gross anatomical studies do not show any dissectable branches from S2 to the middle hemorrhoidal nerve (fig. 1, 2). This apparent discrepancy might be explained on the basis that the afferent fibers from S2 weave proximally and distally in the sacral plexus to reach the middle hemorrhoidal nerve. Since this plexus is formed in the embryo, shifting the anastomoses between nerves during the formation of a plexus does not seem unlikely.

Stimulation of the distal cut end of the middle hemorrhoidal nerves in the majority of the animals resulted in a contraction of the coccygeus, the retractor anus (fig. 10), the external anal sphincter, and the retractor penis muscles. In some instances only the retractor penis muscle contracted. This established that the retractor penis muscle is innervated by both the dorsal nerve of the penis and the middle hemorrhoidal nerve. These experiments indicate

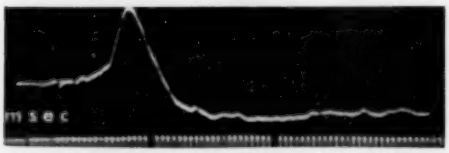


Fig. 7—Compound action potential in dorsal root of S3 after stimulation of the pelvic nerve.

that the middle hemorrhoidal nerve contains sensory nerve fibers, nerve fibers to striated muscle, and sympathetic fibers to the retractor penis muscle.

The caudal hemorrhoidal nerve has a variable origin. In approximately one third of the specimens, it was absent. When present, it arose from S4 in about one half of the animals and from S5 in the other half. Its peripheral distribution overlapped with that of the middle hemorrhoidal nerve.

Middle Route of Nerves Supplying the External Genitalia.—The middle route for the peripheral nerve supply to the genitalia consists of the inguinal nerve. This nerve has been called the external spermatic nerve by a number of authors.[4,7,23] Larsell[14] refers to the external spermatic nerve as one of the two branches of the genitofemoral nerve in man. He states that it supplies the cremaster muscle, as well as the integument of the scrotum. It seems inappropriate to refer to the inguinal nerve as the external spermatic nerve, particularly in the female. It would be desirable to refer to it as the genitofemoral nerve, as recommended by the "Nomina Anatomica"[3] and as Martin and Schauder[19] do. Its peripheral distribution in the ox does not include a branch, the lumboinguinal,[3] as is found in man and which supplies the skin on the medial surface of the thigh. For these reasons we have decided to call this nerve, as proposed by St. Clair,[24] the inguinal nerve.

This nerve, according to St. Clair[24] and Arnold and Kitchell,[1] originates from a plexus formed by nerves from the ventral branches of L2 to L4, the third lumbar component being constant and the second and fourth lumbar contributions being variable. Two nerves emerge from the plexus. One of these passes above and one below the deep circumflex iliac artery. In the bull, these nerves pass through the inguinal canal on the lateral surface of the cremaster muscle (fig. 2). There are anastomotic branches between the two components (cranial and caudal inguinal nerves of St. Clair[24]) in all cases. In several instances, the two nerves were fused as a single entity.

The cranial inguinal nerve supplies the cremaster muscle and terminates in the skin of the fold of the flank. The caudal inguinal nerve also supplies the cremaster

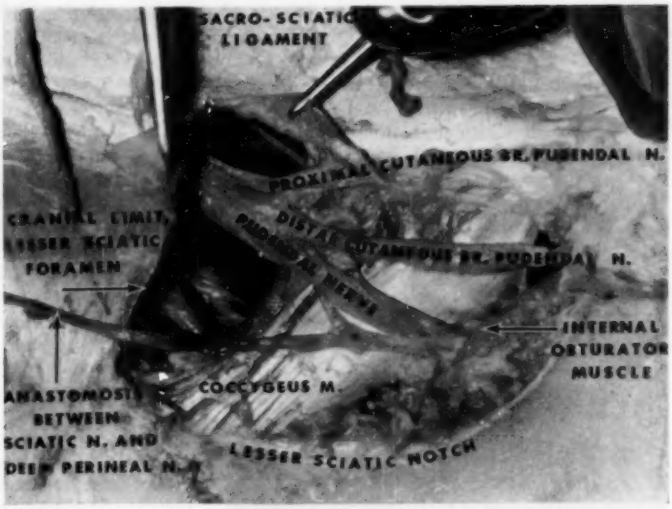

SACRO-SCIATIC LIGAMENT
PROXIMAL CUTANEOUS BR. PUDENDAL N.
CRANIAL LIMIT, LESSER SCIATIC FORAMEN
DISTAL CUTANEOUS BR. PUDENDAL N.
INTERNAL OBTURATOR MUSCLE
COCCYGEUS M.
ANASTOMOSIS BETWEEN SCIATIC N. AND DEEP PERINEAL N.
LESSER SCIATIC NOTCH

Fig. 8—Photograph of dissection showing relations of the pudenal nerve at the lesser sciatic foramen.

muscle, then sends cutaneous branches as far cranially as the preputial orifice (fig. 3). These nerves will be called the middle preputial cutaneous nerves, in contrast to the caudal and cranial preputial cutaneous nerves. The caudal inguinal nerve also gives rise to the lateral scrotal nerves which supply the lateral scrotal wall (fig. 3). In addition, small cutaneous branches supply the immediately adjacent skin.

No systematic exploration of the central afferent connections of the inguinal nerve was attempted. However, it was found that stimulation of the central cut ends of either the cranial or caudal inguinal nerves resulted in a reflex contraction of the cremaster muscle, the caudal fasciculi of the cutaneous trunci muscle, and the protractor and retractor preputiae muscles. If strong stimuli were used, the entire cutaneous trunci muscle contracted. The details of this reflex will be reported elsewhere.[18]

Stimulation of the distal cut ends of the cranial and caudal inguinal nerves distal to the inguinal canal did not result in any contraction of the striated muscles. Stimulation of the plexus forming the inguinal nerve resulted in a contraction of the caudal fascicles of the internal abdominal oblique muscles and, more particularly, the cremaster muscle (fig. 11).

These results indicate that the inguinal nerve is a mixed nerve, proximally, but that its distal part is cutaneous in function.

Cranial Route of Nerves Supplying the External Genitalia.—The cutaneous branches of the ventromedial branches of T9 to T12 and L1 and L2 are distributed to the genitalia of the ventral midline (fig. 3). The branches of T9, T10, and T11 go through the protractor preputiae muscle and are sensory to the skin in the immediate area of this muscle. The branches of T12 are the ones most directly distributed to the preputial orifice area (fig. 3).

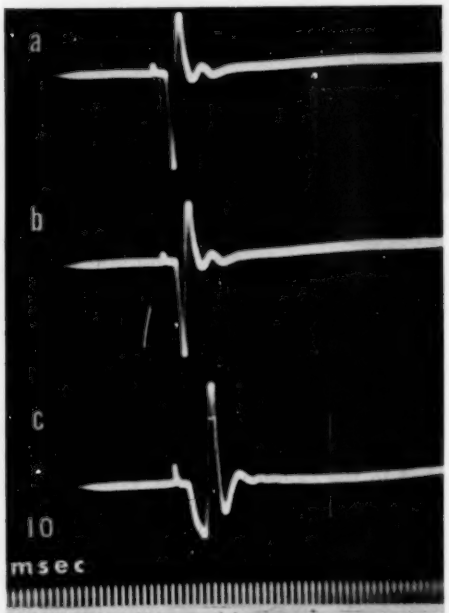
Fig. 9—Photograph illustrating anastomosis between the sciatic and deep perineal nerves. The right of the photograph is ventral.

Branches come off T13, L1, and L2, near their emergence from the abdominal tunic, and go to skin ventral to the prepuce. Each gives off a distinct branch which is distributed to the preputial membrane and the perputial orifice. Collectively, these branches are named the cranial preputial nerves.

Stimulation of the central cut ends of the cranial preputial nerves resulted in a reflex contraction of the cutaneous trunci, protractor preputii, and retractor preputii muscles. No attempt was made to explore the dorsal rootlets of spinal segments involved in each cutaneous branch. That there may be considerable overlap is suggested by the findings of Arnold and Kitchell,[1] which indicate that three spinal nerves may supply a single area of skin on the abdominal wall.

Stimulation of the distal cut ends of the cranial preputial nerves did not result in any contraction of striated muscle. These results indicate that these branches are principally sensory in function.

Branches from the lateral thoracic nerve which arise from the ventral branches of C8, T1, and T2 were observed to go to the protractor and retractor preputiae muscles (fig. 3). The lateral cutaneous rami of the intercostal nerves consistently anastomosed with the lateral thoracic nerve. This made interpretation of these findings impossible, on the basis of gross anatomical observations alone. Results of electrophysiological studies, to be presented in greater detail elsewhere,[18] show that the lateral thoracic nerve is the motor nerve supply to these muscles. The cutaneous rami of the intercostal nerves which join the lateral thoracic nerve are sensory in function.[18]



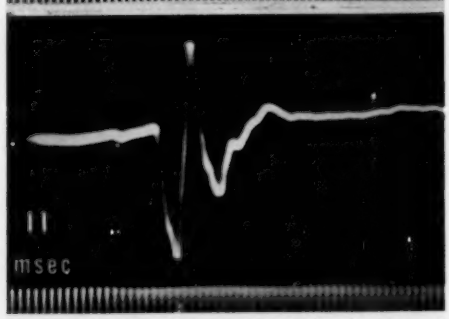


Fig. 10—Electromyographic activity in the (a) anus, (b) retractor anus, (c) external anal sphincter muscles following stimulation of the middle hemorrhoidal nerve of a bull calf.

Fig 11—Electromyographic activity in the cremaster muscle following stimulation of the inguinal nerve of a bull calf.

SUMMARY AND CONCLUSIONS

The combined gross anatomical and electrophysiological studies reported here have been concerned with elucidating the nerve supply to the external genitalia of the bull and the ram.

It was found that the central origin of nerves concerned with supplying the external genitalia can conveniently be divided into cranial, middle, and caudal parts. The cranial part is composed of C8, T1, and T2 (lateral thoracic nerve), T9 through T13, and L1 and L2 (cutaneous rami of ventromedial branches of spinal nerves). The middle portion is made up of L2, L3, and L4 (inguinal nerve). The caudal part is made up of S1 through S5 (pudendal, middle hemorrhoidal, caudal hemorrhoidal, and anastomosis between sciatic and deep perineal nerves).

The following outline is a summary of the nerve supply to the external genitalia according to the various anatomical parts.

1) Prepuce, Including Free Preputial Membrane (All Sensory Nerves).—*(a)* the cranial preputial nerves rise from the

ventromedial branches of T13, L1, and L2; *(b)* the middle preputial nerve from the caudal branch of the inguinal nerve; and *(c)* the caudal preputial nerves from the superficial perineal branch of the pudendal nerve.

2) Protractor and Retractor Preputiae Muscles.—The motor nerve supply to these muscles is via the lateral thoracic nerve from the brachial plexus.

3) Scrotum (All Sensory Nerves).—*(a)* The lateral scrotal nerve, a terminal branch of the caudal inguinal nerve, is distributed to the lateral scrotal wall; *(b)* the middle scrotal nerve, a terminal branch of the superficial perineal nerve (pudendal) supplies the median scrotal septum and the cranial aspect of the corresponding half of the scrotum; and *(c)* the caudal scrotal nerve, formed by the joining together of the distal cutaneous branches of the pudendal nerve, is distributed to the caudal wall of both halves of the scrotum.

4) Perineum and Laterally to the Lateral Boundary of the Semitendinosus Muscle (All Sensory Nerves).—These are *(a)* the proximal cutaneous branch of the pudendal nerves; and *(b)* the distal cutaneous branch of the pudendal nerve, including branches from the caudal scrotal nerve.

5) Body and Glans of the Penis.—*(a)* The sensory nerve to these structures is the dorsal nerve of the penis.

6) Retractor Penis Muscle.—*(a)* The proximal part is supplied by the terminal part of the middle hemorrhoidal nerve, and *(b)* the distal part is supplied by the dorsal nerve of the penis.

7) The Ischiocavernosus, Bulbocavernosus, and External Anal Sphincter Muscles.—These muscles are supplied by the deep perineal nerve which is formed from the pudendal and the anastomotic branch from the sciatic nerve to the deep perineal nerve. The external anal sphincter in some animals also is supplied by the middle hemorrhoidal nerve.

8) The Coccygeus and Retractor Anus Muscles.—These muscles have a variable nerve supply from the pudendal, middle hemorrhoidal, and the deep perineal nerves.

9) Terminal Rectum and Skin Surrounding the Anus (Sensory).—The caudal and middle hemorrhoidal nerves supply this area.

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A Study of Haemonchus in Cattle and Sheep

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IT HAS BEEN CUSTOMARY for parasitologists to record the common large stomach worm of cattle and sheep as *Haemonchus contortus*.

Dikmans [6] and Mönnig,[7] however, reported that a majority of female Haemonchus recovered from sheep had a well-developed linguiform cuticular process covering the vulva, whereas a majority of the females recovered from cattle had a small, knoblike process. This was confirmed by Roberts,[10] who also found that the majority of females recovered from sheep given infective larvae of bovine origin had the knoblike process typical of females from cattle and that the majority of females recovered from cattle given infective Haemonchus larvae of ovine origin had the long linguiform process typical of females from sheep.

Further studies [11, 12] indicated that sheep were susceptible to infection with Haemonchus from cattle, while infections were not readily established in cattle with infective larvae of Haemonchus from sheep. It also was observed [12] that infective larvae cultured from eggs in cattle feces differed in size and activity from those cultured from Haemonchus eggs in sheep feces. Investigations on the cross transmission of these gastrointestinal nematodes showed [9] that calves acquired appreciably more Haemonchus of cattle origin than did lambs, while lambs acquired appreciably more Haemonchus of sheep origin.

Recently, Roberts *et al.*[13] reported that the mean values for spicule length and for the distances between the hooks and the tips of the spicules differed significantly between males recovered from cattle and sheep. As a result of (1) the apparent difference in susceptibility of the hosts to infection with bovine and ovine Haemonchus, (2) the difference in size and activity of infective larvae cultured from eggs passed by cattle and sheep, and (3) the differences in mean values for spicule measurements, these workers concluded that the large common stomach worms occurring in cattle and sheep constitute two distinct species. Consequently, they retained the name *H. contortus* (Rudolphi, 1803) for the species normally parasitic in sheep and applied the name *Haemonchus placei* (Place, 1893) to the species normally parasitic in cattle.

Subsequently, cytological studies made on adult specimens recovered from naturally and experimentally infected cattle and sheep have yielded chromosomal evidence favoring the elevation of bovine and ovine strains of Haemonchus to specific status.[1] The life cycle of *H. placei* in cattle was studied [3] and compared with the life cycle of *H. contortus* in sheep.[15] There appeared to be no difference in the morphology of the comparable parasitic larval stages; however, the prepatent period of *H. placei* was about 11 days longer than that of *H. contortus*.

An investigation [5] of the comparative morphology of infective larvae of worms from beef cattle in Kansas revealed that the infective larvae of bovine Haemonchus apparently were larger than those of ovine origin, thus supporting the Australian workers. However, it was decided [5] that the nematodes be considered one species, pending studies of the adult worms. It has been found [14] that variations in the rations of sheep significantly affected the size of the larval offspring of Haemonchus present in them. Thus, difference in larval size could constitute a questionable criterion for separating Haemonchus from cattle and sheep into two distinct species. However, difference in larval size was observed when both bovine and ovine strains of infective larvae were fed simultaneously to the same lamb,[8] thus negating nutritional influence. Nevertheless, eggs that produced infective larvae typical of *H. contortus* were recovered, 19 days after infection, whereas 11 more days elapsed before eggs that produced larvae typical of *H. placei* were recovered.

In view of these facts, a morphological study was made of the worms recovered and preserved by Porter,[9] and other related, hitherto unpublished, data were summarized to determine whether a similar situation obtains in regard to Haemonchus in cattle and sheep in the United States.

MATERIALS AND METHODS

The worms used in this study were recovered at necropsy from some calves and lambs that had been experimentally infected or from others that had acquired their infections naturally by grazing on two pastures adjoining the Regional Laboratory.[9] Pasture A had been contaminated previously by sheep given infective larvae of the ovine strain of Haemonchus, whereas pasture B had been contaminated by calves given infective larvae of the bovine strain.[9] Both strains had been obtained originally by culturing eggs liberated from females recovered at necropsy from cattle and sheep. The term "strain" as used in this paper denotes the source of the Haemonchus being discussed.

Worms were recovered and counted by the tech-

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TABLE 1—Measurements (μ) of Spicules and Distribution of Types of Vulval Processes of Haemonchus Recovered from Naturally Infected Calves and Lambs

Group (No.)	Host Type	Host No. animals	Haemonchus strain	No. males examined	Mean length of spicules	Mean distance from hook to tip of right spicule	Mean distance from hook to tip of left spicule	No. females examined	Percentage of females with indicated types of vulval process A,B,C	D,E	F to N
1	Lambs	9	Ovine	533	410 ± 17.9	43 ± 3.1	22 ± 2.2	547	64	4	32
2	Calves	8	Ovine	403	414 ± 27.3	43 ± 5.1	23 ± 3.0	558	62	3	35
3	Lambs	6	Bovine	88	453 ± 20.2	54 ± 4.3	27 ± 3.8	94	13	68	19
4	Calves	9	Bovine	492	459 ± 17.5	53 ± 3.5	28 ± 2.7	516	13	79	8
Values of "t"* determined on basis of difference between means of groups											
1 and 2					2.7	0.0	5.9				
3 and 4					2.7	2.5	3.2				
1 and 3					20.3	29.0	19.2				
2 and 4					29.8	34.7	26.2				

* If "t" is > 2.8, P = < 0.01.

niques described by Porter.[6] They were fixed in 70 per cent alcohol and preserved in a mixture composed of 85 parts of 75 per cent alcohol, 10 parts of 10 per cent formalin, and 5 parts of glycerin.

The prepatent period of infection was determined by routine examination of the feces, using the direct centrifugal flotation technique, with zinc sulfate as the flotation solution.

Worms were measured by projecting the shadow of individual worms upon the ground glass of a camera, tracing the enlarged (magnified 4 diameters) shadow on paper, and determining the total length by the aid of a planimeter. Measurements were made of the three spicule characteristics used by Roberts *et al.*[15] as partial criteria for differentiating between the species, *i.e.*, the length of the spicules and the distance from the hook to the tip of each spicule. All measurements were recorded in microns.

The structure or configuration of the vulval process was determined and, on the basis of the types of processes figured by Roberts *et al.*[15] and the designations used by those authors, the females were grouped as follows: (1) those with types A, B, or C, (2) those with types D or E, and (3) those with any of the other nine types (F–N).

Blood samples were taken weekly from the jugular veins, and collected into tubes containing ammonium and potassium oxalate. Hematocrit measurements were determined by centrifuging 1 ml. of blood in Wintrobe tubes at 3,600 r.p.m. for one hour.

RESULTS

Spicules and Vulval Process.—The data pertinent to the measurements of the spicules and to the configurations of the vulval processes are shown (tables 1, 2). In the naturally infected animals (table 1), the mean measurements of the spicule length and of the distances from the hooks to the tips of the spicules in worms of the bovine

TABLE 2—Measurements (μ) of Spicules and Distribution of Types of Vulval Processes of Haemonchus Recovered from Experimentally Infected Calves and Lambs*

Host	Haemonchus strain	Mean length of spicules	"t"†	Mean distance from hook to tip of right spicule	"t"	Mean distance from hook to tip of left spicule	"t"	Percentage of females with indicated types of vulval process A,B,C,	D,E	F to N
C-118	Bovine	478 ± 17.5	0.0	57 ± 4.5	0.0	28 ± 3.5	2.3	30	66	4
L-5	Bovine	478 ± 11.9		57 ± 3.0		29 ± 2.6		29	63	8
C-249	Bovine	462 ± 16.3	0.5	52 ± 2.6	2.3	26 ± 3.2	7.1	26	65	9
L-213	Bovine	461 ± 13.8		53 ± 3.3		29 ± 2.8		18	74	8
C-250	Bovine	479 ± 16.5	7.2	57 ± 3.5	2.0	29 ± 2.6	2.7	28	72	0
L-219	Bovine	463 ± 14.2		56 ± 3.5		28 ± 2.6		21	68	11
C-117	Ovine	438 ± 14.7	0.5	45 ± 3.3	3.9	22 ± 2.2	1.0	84	0	16
L-6	Ovine	439 ± 13.6		43 ± 3.8		21 ± 2.3		83	0	17
C-169	Bovine	483 ± 15.0	21.6	57 ± 3.1	22.9	29 ± 2.6	17.1	38	59	3
C-170	Ovine	436 ± 15.8		46 ± 3.7		23 ± 2.4		96	4	0
C-271	Bovine	466 ± 15.0	23.3	55 ± 4.4	20.8	28 ± 2.6	15.2	21	75	4
C-272	Ovine	413 ± 17.1		44 ± 3.0		23 ± 2.1		62	1	37

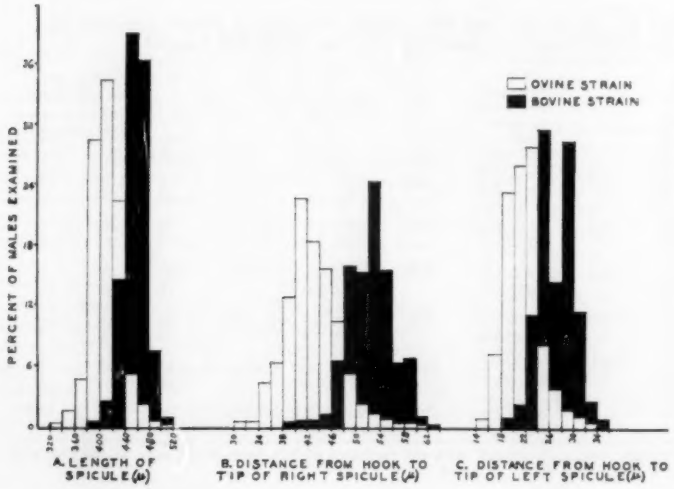
* 100 male and 100 female worms from each animal, with the exception of L-5, from which only 65 females were examined; † if "t" is > 2.8, P = < 0.01.

strain differed markedly and consistently from the means of the ovine strain worms, regardless of the ultimate host type. Lot means of the bovine and ovine strains ranged as follows: spicule length, from 454 to 470 μ and 398 to 431 μ, respectively; distance between hook and the tip of right spicule, from 52 to 54 μ and 41 to 46 μ, respectively; and distance between hook and the tip of left spicule, from 27 to 30 μ and 21 to 24 μ, respectively. Frequency distributions of the spicule measurements showed some overlapping of individual values (graph 1, A-C). Essentially the same situation prevailed for the worms recovered from the experimentally infected calves and lambs (table 2); *i.e.*, there were consistent, marked differences in mean spicular measurements between worms of the two strains, regardless of the ultimate host and irrespective of the generation number of the infective larvae. On the other hand, in worms of the same strain recovered from the two host types, the mean measurements were the same or differed only slightly.

When analyzed statistically, using the "t" test for significance, the mean differences between strains were all highly significant at the 1 per cent level. Differences between worms recovered from calves and lambs infected with the same strain were significant in a few instances, but never in more than one of the three spicular criteria when any two specific worm populations were compared. For example, when the mean measurements of the spicules of bovine strain Haemonchus from calf 249 and lamb 213 are compared, only the difference in mean distance from the hook to the tip of the left spicule is significant, whereas the differences in mean spicule length and in distance from hook to tip of right spicule are not significant.

The vulval process configurations in the female worms of the bovine strain were predominantly those of types D and E (short, knoblike), whereas in worms of the ovine strain, the process was predominantly of types A, B, and C (long, linguiform). However, some worms of both strains had processes typical of the opposite strain or of the nine miscellaneous configurations (F–N).

Length of Worms.—Both sexes of bovine strain worms from calves had mean lengths greater than those of worms of the same strain from lambs. The opposite was true of the ovine strain. The differences between mean lengths of worms from each experimental host-pair (table 3) are highly significant at the 1 per cent level, excepting the difference between the mean length of males from calves 271 and 272, where the difference approaches significance at the 2 per cent level. When all data were pooled, for all measured worms of each strain from the two host types, worms of each strain from the normal host had significantly greater mean lengths than those of the worms from the opposite host. When all measured bovine strain Haemonchus from calves were compared with all


OVINE STRAIN
BOVINE STRAIN
PERCENT OF MALES EXAMINED
A. LENGTH OF SPICULE (μ)
B. DISTANCE FROM HOOK TO TIP OF RIGHT SPICULE (μ)
C. DISTANCE FROM HOOK TO TIP OF LEFT SPICULE (μ)

Graph 1—Histograms of the frequency distribution of measurements of the spicules of male Haemonchus from sheep and cattle.

measured ovine strain worms from lambs, the differences in mean lengths were not significant.

Prepatent Period, Number of Worms Recovered, and Blood Picture.—The prepatent period of infection of the bovine strain ranged from 24 to 28 days in 5 calves and from 31 to 32 days in 3 lambs, whereas the prepatent period of the sheep strain was 18, 20, and 21 days, respectively, in 3 calves and 22 days in 1 lamb.

The data show that more worms were recovered from each of 3 calves than from each of 3 lambs when all 6 animals were infected with the bovine strain. The opposite was true of a calf and a lamb infected with the ovine strain. More worms were recovered from calf 271 that had been infected with the bovine strain than from its mate, calf 272, that had been given an equal number of infective larvae of the ovine strain, but this situation was reversed in another pair of calves (No. 169, 170). Pooled data showed (table 3) that a larger percentage of each strain was recovered from the normal host than from the abnormal one.

The data for 3 calves and 3 lambs given bovine strain infective larvae showed (graph 2, A,B,C) a consistent reduction in packed erythrocyte volume in each calf, but essentially no change in the volume for the lambs. Results for the calf and lamb given ovine strain larvae were surprising, since the packed cell volume (graph 2, D) was reduced considerably in the calf, although it harbored (table 3) only about one eighth as many worms as the lamb at necropsy; however, cell volume was more severely reduced in the lamb, which ultimately died of its infection. Packed cell volumes (graph 2, E) were reduced almost identically in calves 271 and 272, given the same number of larvae of the bovine and ovine strains, respectively, and harbored somewhat comparable numbers of worms at necropsy. However, the packed cell volume (graph 2, F) was only slightly reduced in calf 169, given bovine strain infective larvae, whereas the packed cell volume was sharply reduced in calf 170, given an equal number of ovine strain larvae. The latter calf harbored nearly four times as many worms as the former at necropsy.

DISCUSSION

These data confirm the observations of Roberts *et al.*,[13] in that the mean measure-

TABLE 3—Numbers of Worms and Body Length Measurements (mm.) of Bovine and Ovine Strain Haemonchus Recovered from Experimentally Infected Calves and Lambs

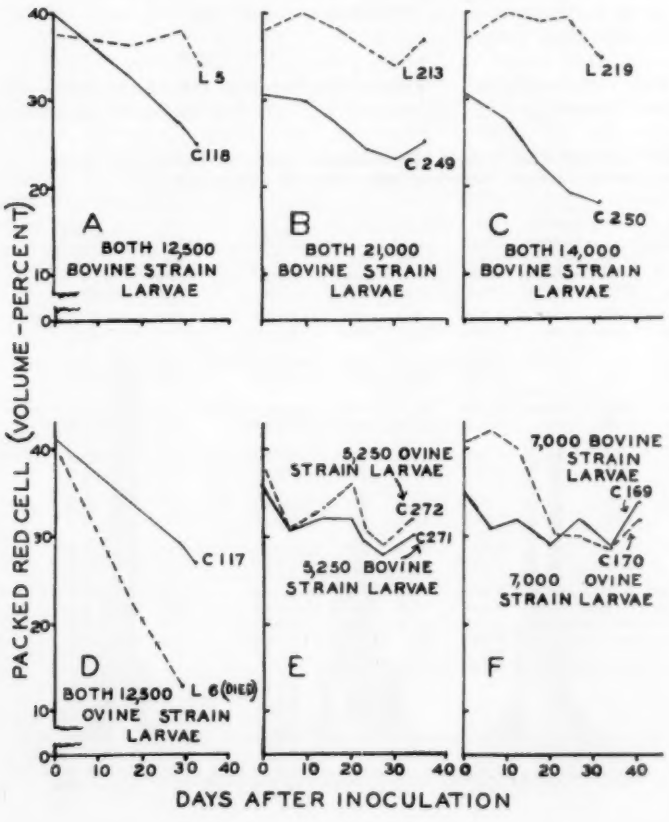
Host		Infective larvae administered			Interval between inoculation and necropsy (days)	No. worms recovered	Percentage of larvae administered	Males			Females		
Animal (No.)	Age (weeks)	No.	Strain	Generation				No. examined	Mean length	"t"*	No. examined	Mean length	"t"
C-118	4	12,500	Bovine	9th	35	1,237	9.9	169	14.2±1.5	37.2	220	18.2±1.7	32.1
L-5	8½	12,500	Bovine	9th	32	283	2.3	183	9.6±1.0		78	11.6±1.2	
C-249	10	21,000	Bovine	13th	37	7,477	35.6	100	14.8±0.9	20.2	100	18.7±0.4	18.5
L-213	11	21,000	Bovine	13th	37	5,163	24.6	100	12.4±0.9		100	15.4±1.4	
C-250	10	14,000	Bovine	13th	32	7,249	51.8	100	14.9±0.7	27.7	100	19.5±1.0	19.9
L-219	11	14,000	Bovine	13th	32	3,002	21.4	100	11.8±0.9		100	15.7±1.6	
C-117	4	12,500	Ovine	8th	35	1,069	8.6	187	13.0±1.0	9.4	147	17.4±1.5	10.9
L-6	8½	12,500	Ovine	8th	32	8,505	68.0	377	14.0±1.3		166	19.7±2.1	
C-169	8	7,000	Bovine	11th	44	882	12.6	100	15.2±1.2	10.2	100	19.7±1.8	2.8
C-170	8	7,000	Ovine	9th	44	3,028	43.3	100	13.3±1.4		100	19.0±1.8	
C-271	19	5,250	Bovine	15th	35	2,444	46.6	100	14.4±0.9	2.4	100	19.2±0.9	5.1
C-272	19	5,250	Ovine	15th	35	1,338	25.5	100	14.1±0.9		100	18.4±1.2	
Group 1, all ovine strain worms recovered from lambs						8,505	68.0	377	14.0±1.3		166	19.7±2.1	
Group 2, all ovine strain worms recovered from calves						5,435	22.0	387	13.4±5.2		347	18.1±6.8	
Group 3, all bovine strain worms recovered from lambs						8,448	17.8	383	10.9±6.2		278	14.4±9.0	
Group 4, all bovine strain worms recovered from calves						19,829	33.2	569	14.4±6.0		620	18.9±6.1	
Values of "t" determined on basis of difference between means of groups													
1 and 2									2.7			2.8	
1 and 3									7.5			6.1	
2 and 3									6.0			5.8	
2 and 4									2.8			4.2	
3 and 4									8.7			8.7	
1 and 4									1.1			1.4	

* If "t" is >2.8, P = <0.01.

ments of spicule length and the distances from the hooks to the tips of the right and left spicules differ between worms of the bovine and ovine strains of Haemonchus, with these means being greater in the bovine strain. The range of lot means observed in our study is in close agreement with the range reported by those authors. The results of this study suggest that the differences in spicule measurements between the two strains reflect some form of inherent control rather than hostal influence, as these means remained relatively constant for each strain, regardless of whether its ultimate host was cattle or sheep, whereas the length of the worms of each strain was stunted by the opposite host. Lack of influence of host on spicule size is indicated further by the fact that significant differences in mean measurements of spicules were evident between bovine strain worms recovered from calves and ovine strain worms recovered from lambs, whereas the difference in mean length of the worms was insignificant.

There was considerable overlapping of individual spicule measurements; consequently, it would be difficult, if not impossible, to determine the strain of an individual male specimen. However, bracketing, by groups of 10, all measured worms of each strain from each of the naturally and experimentally infected animals revealed that the mean for each group, consisting of only 10 specimens, fell without exception within the appropriate range of the recorded lot means.

A preponderance of bovine strain females examined had short, knoblike vulval processes, whereas a majority of the ovine strain females had the long, linguiform type. Although this is in agreement with the observations of Roberts *et al.*,[13] a greater percentage of worms of each strain had vulval processes typical of the opposite strain or of the miscellaneous types



Graph 2—Hematocrit values for lambs and calves infected with Haemonchus from cattle and sheep.

than they recorded. Possibly some of the atypical vulval configurations observed in our study were the result of some type of adverse effect exerted by the abnormal host. It has been reported [6] that, when *Ostertagia ostertagi*, a nematode normally parasitic in cattle, is recovered from sheep, the females lack the typical, highly distinctive vulval flap almost invariably present on worms recovered from the normal host.

The numbers of worms recovered were variable, as were the percentages of larvae administered that these numbers represented. In general, more worms were recovered at necropsy from calves infected with the bovine strain than from lambs infected with the same strain, whereas the opposite was true for the ovine strain. However, 1 calf given ovine strain larvae harbored four times as many worms as another calf simultaneously given an equal number of bovine strain larvae. It is probable that this apparent reversal from the normal was a result of an abnormal resistance or susceptibility in these 2 calves. Other studies [11,13] have indicated that the bovine strain of Haemonchus can be established readily in sheep, while it is difficult to establish the ovine strain in cattle. The results of our study are not in agreement with this, as the ovine strain appeared to establish itself in calves as successfully as the bovine strain in sheep. Perhaps this divergence in results may be attributed to the difference in the duration of the infections, as animals in our study were killed from 32 to 44 days after administration of infective larvae, whereas the interval between acquisition of infective larvae and necropsy was from 70 days to considerably longer in the work reported by Roberts [11] and Roberts *et al.*[13]

Recently, Chitwood [3] concluded, from a perusal of the paper by Roberts *et al.*[13] and from a study of Haemonchus specimens from various ruminants, that (1) it is possible that conditions in Australia are such that "separation of the sheep and cattle types of *H. contortus* may eventually result in differences at the species level," and (2) "that the Haemonchus in Australia may differ from the Haemonchus found in both domestic and wild ruminants in the United States." However, on the basis of our general agreement with Roberts *et al.*,[13] in reference to spicule measurements and the relative ability of the two strains to infect cattle and sheep, and our agreement with Bremner [2] on the difference in prepatent periods between the two strains, we are of the opinion that the two strains of Haemonchus involved in this study are comparable to the two species of Haemonchus advanced by the Australian workers, *i.e., H. placei* and *H. contortus*.

Summary and Conclusions

1) A study was made of 2,716 male and 2,880 female Haemonchus recovered from cattle and sheep that acquired their infections by grazing on pastures contaminated with Haemonchus of bovine or ovine origin, and from other animals that had been experimentally infected.

2) The mean measurements of spicule length and of the distances from the hooks to the tips of the spicules of the bovine strain were significantly greater than those of the ovine strain. The differences in spicule measurements between the two strains were present regardless of the ultimate host, indicating that mean measurements of spicules were not affected by the host.

3) Of the bovine strain females examined, 72 per cent had a short, knoblike vulval process, 20 per cent had a long, linguiform process typical of the ovine strain, and 8 per cent had processes with other configurations. However, 68 per cent of the ovine strain females examined had a long, linguiform process, 3 per cent a short knoblike process, and 28 per cent had vulval processes with miscellaneous configurations.

4) Bovine strain worms recovered from experimentally infected lambs were significantly shorter than those recovered from similarly infected calves. The opposite was true of the ovine strain.

5) More worms were harbored by calves experimentally infected with the bovine strain than by lambs similarly infected, whereas the opposite was true for the ovine strain.

6) Limited blood studies indicated that ovine strain Haemonchus caused a reduction in packed erythrocyte volume in both lambs and calves, while the bovine strain worms reduced packed cell volume markedly in calves but apparently had little effect on cell volume in lambs.

7) The prepatent periods of infection differed between the two strains.

8) The evidence indicates that the two strains involved in this study were comparable to the two species of Haemonchus advanced by the Australian workers. *i.e., H. placei* and *H. contortus,* normal parasites of cattle and sheep, respectively.

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Morphological and Histochemical Study on the Ovary of the Sheep

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PAPERS on the histology of the sheep ovary have been published by Haller and Kuehlemann (1754),[17] Paterson (1840),[32] Marshall,[26,27] Warbitton,[36] Robinson,[34] and others. It was felt that there are many aspects of ovarian histology which have not been adequately described. No paper has been written on the histochemical characteristics of the sheep ovary. Therefore, in addition to morphological studies, a series of common histochemical tests was attempted, to establish the type of histochemical reactions obtainable in the ovine ovary.

MATERIALS AND METHODS

The genital tracts of 60 Scottish Blackface ewes were used. A previous paper was devoted to the description of the oviduct secretion in the same animals[15] and another one to the histochemical reactions in their uteri.[15]

The specimens represented a complete sexual cycle. Ten of the genitalia were obtained from multiparous experimental ewes which were kept on a college pasture with a teaser ram and had shown estrus regularly for at least two consecutive cycles before the specimens were obtained. The material from these 10 experimental animals was recovered through hystero-oophorectomy and was put into the required fixatives immediately.

The remaining material was obtained at the Glasgow abattoir from 50 freshly slaughtered sheep. The correct phase of the cycle in the abattoir ewes was estimated from the gross anatomic and microscopic appearance of the genital tract and ovaries, and was compared to those obtained from the experimental animals. These 50 specimens were collected to serve as controls, and to ensure that the comparatively low number of specimens is not misleading.

After recovery, the ovaries were cut into 3- to 5-mm.-thick portions for fixation. Fixatives used for morphological studies were Regaud's solution,[9] Bouin's fluid,[9] and formol-saline solution.[9] Paraffin sections were cut serially from a large number of blocks, at an average of 6 μ, and frozen sections were cut at 10 to 20 μ. All microscopic measurements were performed on sections fixed with Bouin's fluid for comparison with data compiled by Harrison[13,19] and others.

Staining methods used for morphological observations included Harris' hematoxylin and eosin stain,[9] Laidlow's method for reticular fibers,[4] Van Gieson's stain for collagen fibers,[9] and Masson's stain for the different tissue elements.[9]

The histochemical techniques performed can be divided into the following groups:

Inorganic Substances—Iron.—Prussion blue and Turnbull's blue for ferric, ferrous, and organic (masked) iron, Lavollay's hydroxyquinoline test[10] for iron, Humphrey's dinitrosoresorcinol test,[10] and the hematoxylin-lake method (hemosiderin iron)[23] were used.

Organic Substances—Polysaccharides.—The periodic acid-Schiff (PAS) method according to McManus[28,29] and Hotchkiss[12,22] was used. Only occasional sections were counterstained with hematoxylin. If staining with PAS was positive, sections were treated (1) with diastase* for 30 minutes at 37 C. to eliminate the presence of glycogen, (2) with 1,000 units of hyaluronidase (Hyalase†) per 100 ccm. of buffered solution for two hours at 37 C.[16] to establish whether the positive reaction was due to the presence of hyaluronic acid, and (3) with warm methanol-chloroform mixture for 12 hours[24] to determine if the positive reaction was caused by the presence of glycolipid. If controls 1, 2, and 3 were negative (if the material continued to react with PAS after the respective treatment), sections were stained (4) with Southgate's mucicarmine,[9] (5) with toluidine blue, and (6) with celestine blue[11] to test for the presence of mucopolysaccharides.

Lipids.—The Sudan IV stain, according to Herxheimer's technique, and hematoxylin, as in the Lorrain-Smith-Dietrich method,[9] for the general staining of lipids and lipoids,[5,6] were used. Baker's acid hematin test and the pyridine extraction method were used to establish the presence of phospholipids, and Schultz's technique[31] was used to determine if the lipid was cholesterol.

Pigments.—A number of the previously described reactions which were applicable, e.g., iron reaction tests for the presence of polysaccharides and lipids, were used, as well as washing with saturated acids, alkalies, and acidified permanganate according to Gömöri.[11]

Enzymes.—The methods for alkaline glycerophosphatase according to Gömöri[11] were used, with

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* Diastase is produced by the British Drug House, England.

† Hyalase is produced by Benger and Co., England.

an incubation period of five minutes to two hours. The treatment of control slides was identical to that advocated by him. All histochemical reactions were controlled carefully by the use of other tissues for which the glycogen, lipid, or enzyme content was established previously.

THE BREEDING SEASON AND THE SEXUAL CYCLE IN THE EWE

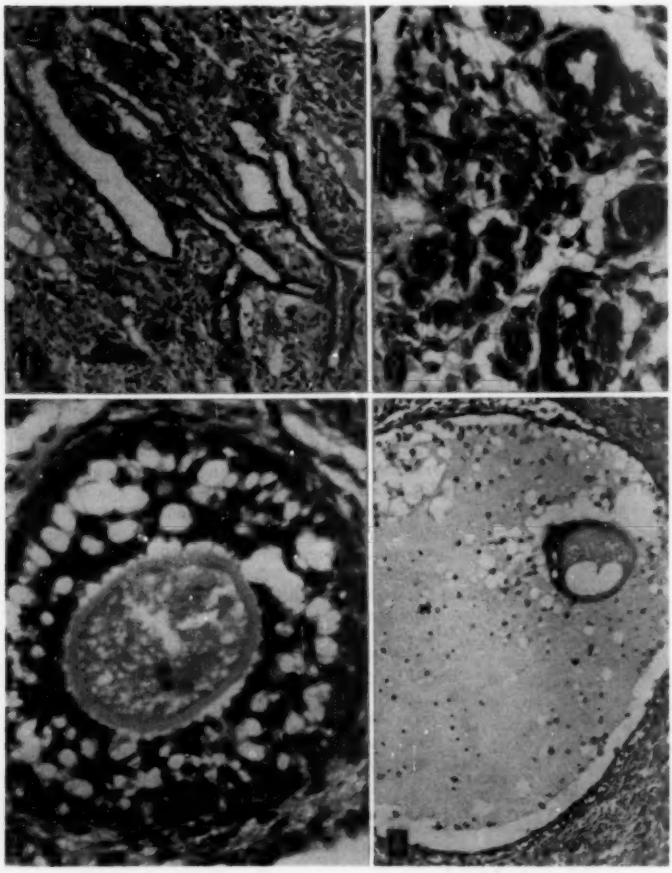
The domestic sheep in Scotland is seasonally polyestrous. The breeding season starts in autumn, but the exact time of its beginning is subject to breed, geographical, and thermal influences. The breeding season in some of the lowland sheep is longer than that of the highland breeds. In the majority of sheep, the sexual season begins in late October. The average length of the cycle is 16.5 days.[12] Estrus, or heat, averages 20 to 24 hours; metestrus, two days; diestrus, nine to 11 days; and proestrus, two to three days.

HISTOLOGY

The ovary of the sheep can be divided readily into a cortical and a medullary layer. There is a thick, collagenous, tunica albuginea beneath the germinal epithelium which is absent from places where corpora lutea and follicles approaching ovulation occupy the ovarian surface.

A framework of reticular fibers extends over the whole ovary and the individual follicles are situated within the branches of this structure.

The vestiges of the rete ovarii often were encountered in the medulla. They usually were small, ductlike structures, circular in transverse sections. Occasionally, however,



Sections of Sheep Ovaries

Fig. 1—Longitudinal section showing the rete ovarii. H & E stain; x 100.

Fig. 2—Transverse section showing the rete ovarii. H & E stain; x 220.

Fig. 3—Contiguous membrana granulosa cells in a developing secondary follicle. H & E stain; x 350.

Fig. 4—Early stage of follicular atrophy. H & E stain; x 75.

they could be seen enlarged in the form of sinusoids. Their lumina were lined by single layers of cuboidal cells (fig. 1, 2).

OOGENESIS AND FOLLICULAR DEVELOPMENT

With regard to the proportion of follicles in different developmental stages, it was found that during anestrus only a low percentage of the total were primary follicles and that the majority were graafian follicles. In ovaries obtained in proestrus, the greater proportion of follicles still remained secondary, although the total follicular number was higher than in anestrus. The greatest number of primary follicles was seen in estrus; they decreased during metestrus, and the diestrous ovary presented a picture similar to that seen during anestrus.

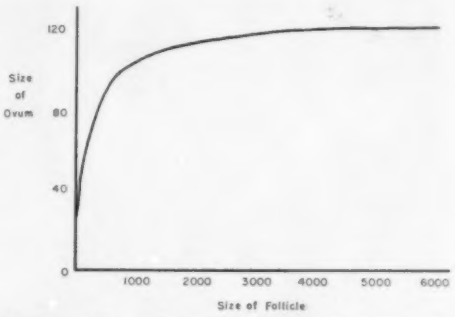
The smallest oocytes, measuring 20.0 to 22.0 μ, were recovered in the outermost layer of the cortex. They were surrounded by flat follicular cells in a primary follicle and by a basement membrane. When the oocyte has reached the size of 28.5 μ, the flat follicular cells become cuboidal. This change was first observed at the two opposite poles of the oocyte. The first appearance of the zona pellucida was seen in a follicle of 66.5 μ, and the first theca interna cells were observed at the same time.

When the follicle reached the size of 80 μ, the cuboidal cells appeared "spongy" and weblike. The individual cells, by means of their processes being in contact with one another, *i.e.*, forming a contiguous structure (fig. 3), thus formed a graafian follicle.

THE GROWTH OF THE FOLLICLE AND OVUM

In 300 follicles of all sizes, the diameters of the follicles and ova were measured. The results were treated statistically to establish the relationship between follicular growth and the growth of the ovum.

Comparison of follicular to oocyte growth shows that, in the early stages of development, the oocyte grew at a rate similar to that of the follicle. In the primary follicles of 26 μ, the oocyte already measured 22 μ. However, when the follicle reached the size of approximately 300 μ, the rate of growth of the oocyte was reduced abruptly, and the follicular growth was more rapid than that of the oocyte thereafter. In the remaining period of growth, the follicle reached the size of 5 to 10 mm., while the oocyte reached the maximum size of only 145 μ.



Graph 1—The comparative growth of ovum and follicle [$Y = 85.4117 + 20.1933$ ($\log_e F = 5.8605$)].

When these measurements are reproduced graphically (the size of the oocytes plotted against the actual follicular size), a steeply rising, almost vertical line appears, which represents the period of relatively rapid growth of the ovum. After this, the line abruptly flattens out into an almost horizontal line which corresponds to the period of relatively slow growth. From all available data, the formula for the regression line for the whole period of growth was $Y = 85.4117 + 20.1933$ ($\log_e F = 5.8605$). Where Y is the size of the ovum, F is the size of the follicle; 85.4117 is the constant and 20.1933 is the coefficient (graph 1).

MULTINUCLEAR OOCYTES, POLYOVULAR FOLLICLES, AND ACCESSORY OOCYTES

Large numbers of polyovular follicles and multinuclear oocytes were observed in the sheep's ovary. These structures were found only in the ovaries of animals killed during proestrus, estrus, and metestrus.

The number of multinuclear oocytes was low at the beginning of proestrus (1.0–2.0%) and high in animals killed at estrus (12.5%). They were found to be degenerating during metestrus and early diestrus. All oocytes with more than one nucleus were in small follicles and no multiple nuclei were found within any of the secondary follicles. Multinuclear oocytes most frequently had two nuclei, but a considerable number had three to five.

The development and atresia of oocytes and follicles was found to be a seasonal process in the ovary of the sheep. The

Fig. 5—Section of a sheep ovary, showing the developing corpus luteum approximately six hours after follicular rupture. H & E stain; x 20.

number of atresic follicles was large during diestrus and small in proestrus.

The degeneration attacked first the smallest and then, gradually, the largest follicles.

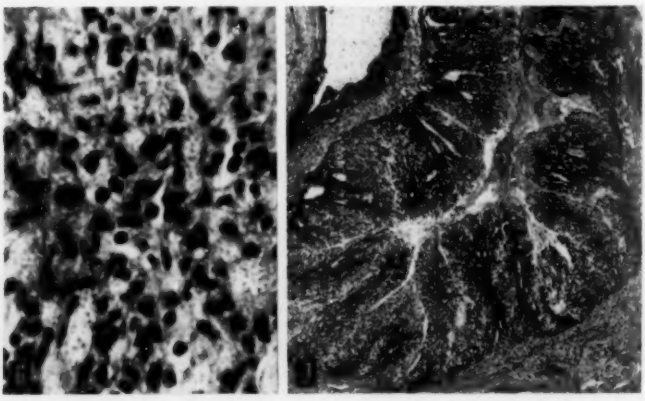
Atresia took different forms in the various stages of development. In primary follicles, the sign of atresia was the disorganization of the vitelline material into a weblike structure, leaving part of the cell devoid of any vitellus. In the more highly developed follicles, the atresia affected the entire follicle at once. The first change was the flattening of the membrana granulosa cells. Second, loosening of pyknotic membrana granulosa cells (fig. 4) and invasion of the follicle by leukocytes occurred. Third, the follicular fluid accumulated into little globules and the oocytes ultimately underwent the same changes as the smaller follicles. When the degeneration was complete, the follicle was filled with connective tissue.

THE CORPUS LUTEUM

A few hours after follicular rupture, the externally visible portion (the ovulation point) appeared as a slightly bleeding small hole, as a slit of varying size, or as a small blood clot.

The 24-hour-old corpus luteum appeared as a small bright red spot, with a scarcely visible opening. In the 2-day-old corpus luteum, a gradual protrusion took place while, in the 3-day-old corpus luteum, the opening was a circular elevation just above the ovarian surface. During the next six or seven days, the color of the corpus luteum changed from red to pink and the growth continued. On the sixteenth day, when a new graafian follicle ruptured, the color of the 1-cycle-old corpus luteum was whitish yellow. After the rupture of the mature follicle, the 16-day-old corpus luteum decreased in size.

It was found that the corpora lutea of different ewes were of almost the same size at identical developmental stages, regardless of the age of the animal or the size of the ovary. A cavity in the center of the corpus luteum often persisted throughout the first 16 days of luteal development. On microscopic examination it was found that, in specimens obtained six to seven hours after ovulation, the theca interna cells had broken through the basement membrane. The membrana granulosa cells had expanded rapidly and occupied an extended area on the periphery. Blood still was visible within the follicular cav-



Sections of Sheep Ovaries

Fig. 6—The developing corpus luteum, approximately 16 hours after follicular rupture. H & E stain; x 400.

Fig. 7—A 36-hour-old corpus luteum. H & E stain; x 40.

ity and between the membrana granulosa cells (fig. 5, 6).

In the 12- to 16-hour-old corpus luteum, groups of granulosa-lutein cells were surrounded by connective tissue trabeculae. The 24- to 48-hour-old corpus luteum appeared completely organized (fig. 7).

After the third day, the connective tissue showed vigorous growth and, by the seventh day, it surrounded every group of luteal cells.

Twelve days after ovulation, at the approach of the next proestrus, the corpus luteum began to decrease in size. This atrophy was a gradual process and began with a decrease in cell size and with pyknosis of the nuclei. The connective tissue trabeculae gradually increased in size and the lutein cells became smaller. The whole process of degeneration gathered momentum when the new estrus started and advanced rapidly after rupture of the new follicle. Strongly developed connective tissue ultimately replaced the lutein cells completely.

By the end of the second cycle, the original corpus luteum was a connective tissue structure well within the ovary. In the center of the organ, a considerable number of large cells with round nuclei and brightly acidophilic cytoplasm were visible; the cytoplasm appeared to be composed of a number of acidophilic globules. These cells decreased in number with each successive ovulation until only a small connective tissue structure remained of the follicle (fig. 8, 9).

Histochemical Reactions in the Ovary

Reactions in the Ovarian Stroma.—No inorganic substance was found in the ovary.

Organic Substances—Polysaccharides.—The basement membranes always reacted positively with PAS and Southgate's mucicarmine stain. The strength of the reaction was not affected by treatment with diastase or hyaluronidase or by the methanol-chloroform extraction, and no metachromasia or other histochemical reaction was detected.

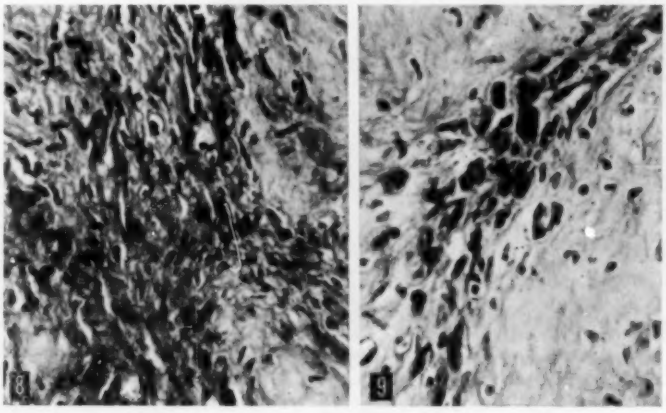
Reactions in the Follicle.—No inorganic substance was found in the ovarian follicle of the sheep.

Organic Substances—Polysaccharides.—Of the follicular components, the zona pellucida and the basement membrane showed continuously strong reactions with PAS, which remained positive even after diastase treatment, and strong metachromasia with metachromatic dyes.

The rest of the follicle provided three distinct phases of reactivity to PAS and metachromatic dyes.

1) In the first phase (follicles under 500 μ in diameter) there were no PAS-positive reactions in the liquor folliculi and the membrana granulosa.

2) In follicles belonging to the second phase (500 μ to 12 mm. in diameter), the liquor folliculi gradually acquired an affinity for and showed strong reactions with PAS. Periodic acid-Schiff-positive granules also developed in the membrana granulosa cells (fig. 10). Both the granules and the liquor folliculi reacted with metachromic dyes. However, the reaction disappeared from both these follicular components after



Sections of Sheep Ovaries

Fig. 8—An atrophied corpus luteum (corpus albicans), 2 cycles (32 days) old. Masson's stain; x 300.

Fig. 9—An atrophied corpus luteum (corpus albicans), 3 cycles (58 days) old. Masson's stain; x 400.

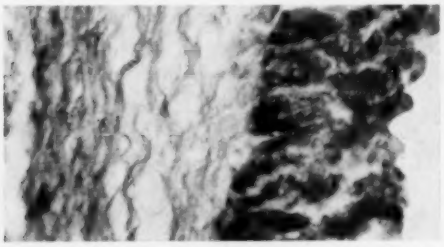


Fig. 10—Section of a sheep ovary, showing the periodic acid-Schiff reaction in the membrana granulosa cells of large graafian follicles, before follicular rupture. x 1,000.

treatment with hyaluronidase. There also were phases of follicular activity when, for no apparent reason, the PAS-positive and metachromatically reacting cell components disappeared. Follicles of every size and in every phase of the cycle were affected by this change.

3) The third phase of follicular affinity toward PAS and metachromatic dyes was observed in atrophying follicles. In these follicles, the globules formed by the liquor folliculi reacted strongly with PAS and metachromatic dyes, and the strength of the reaction was not affected by hyaluronidase, diastase, or methanol-chloroform treatments.

Lipids (Frozen Sections).—Lipid globules were observed in the membrana granulosa and theca interna cells of follicles which measured more than 500 μ. The lipid globules stained with Sudan IV, hematoxylin (Lorraine-Smith-Dietrich method), and Baker's acid hematin. Schultz's cholesterol test was negative. The lipid was extractable by pyridine.

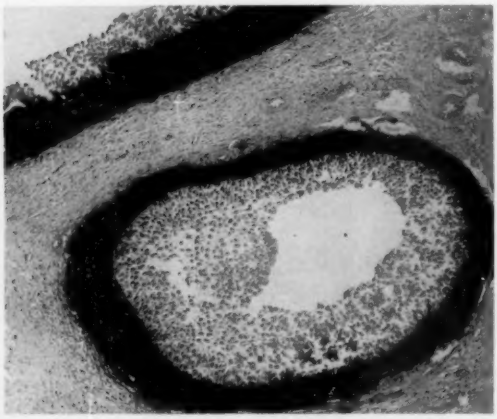


Fig. 11—Section of a sheep ovary, showing the alkaline (nonspecific glycero-) phosphatase reaction in the wall of a graafian follicle. x 40.

Pigments.—No pigments were recovered in the follicle.

Enzymes.—An alkaline (glycero-) phosphatase cytoplasmic reaction was present in the theca interna cells in follicles larger than 500 μ (fig. 11). Occasionally, those layers of the theca externa and membrana granulosa nearest to the theca interna also showed positive reactions.

The whole atrophying follicle, including the liquor folliculi, showed a greatly enhanced alkaline (glycero-) phosphatase reaction.

Reactions in the Corpus Luteum and Corpus Albicans.—No inorganic substance was present.

Organic Substances—Polysaccharides.—Polysaccharides were present only in the cells of the corpora albicantia.

Lipids.—There was an increasing lipid content in the lutein cells which was proportional to the age of the follicle. The maximum lipid content corresponded to the highest luteal development. At that stage, the granulosa-lutein cells were almost completely occupied by lipid and the theca interna cells also contained a large amount. In frozen sections, the lipid was stained by Sudan IV, the Herxheimer technique, the Lorrain-Smith-Dietrich method, and Baker's acid hematin method. Pyridine-extracted sections were negative to acid hematin. They also were negative to the Feulgen plasma and Schultz cholesterol tests.

Although the greater amount of luteal lipid disappeared during the preparation for paraffin embedding, some of the lipid in the theca interna-lutein cells remained and gave a recognizable positive stain with Sudan black B.

Pigments.—Pigment granules were observed only in the cells of the corpus albicans. They were yellow-brown, reacted with lipid stains, and were extremely resistent to fat solvents. Washing with hydrochloric, sulphuric, and acetic acids, alkalies (NOH, KOH, NOOH), or acidified permanganate produced no alterations.

In paraffin-embedded sections, the cells in the corpus albicans stained well with PAS, in addition to staining with Sudan

black B. The strength of these reactions diminished after washing the paraffin sections in acetone. Fat solvents decolorized the sections, but they could be restained with the same dyes (fig. 13).

Enzymes.—At the beginning of corpus luteum formation, the alkaline phosphatase reaction was strong in the theca interna cells, which contrasted sharply with the negative membrana granulosa cells (fig. 12). At about the tenth postovulatory day, the granulosa-lutein cells began to show the presence of alkaline phosphatase, which diminished after two or three days and completely disappeared by the beginning of the next estrus.

Discussion

An uncommon feature in the sheep ovary was the presence of multinuclear oocytes and polyovular follicles. These two structures which, according to Baesich [3,4] are to be regarded as developing under the influence of the same causative agent, have been reported to occur in the human embryo, in the neonatal human being,[3,4] in the cow,[21] in the goat,[18] and in the horse.[19]

In the sheep, as in the goat and the cow, the polyovular follicles increased from a small number at early protestrus to some 12 per cent of the total just after estrus.

The disappearance of the follicular cells which surround the early oocytes was described as a degenerative change in the ovary of the goat. In the ovary of the sheep, however, no degenerative change of the follicular cells was observed, and the opinion was that the cells gradually became transformed into membrana granulosa cells.

The relationship between the growth of the follicle and the growth of the ovum in the sheep's ovary is similar to that in the goat [18] and the sow.[21] The data are comparable to those for the follicular growth in the rat [13] and to comparative measurements on the sow and the cow.[31]

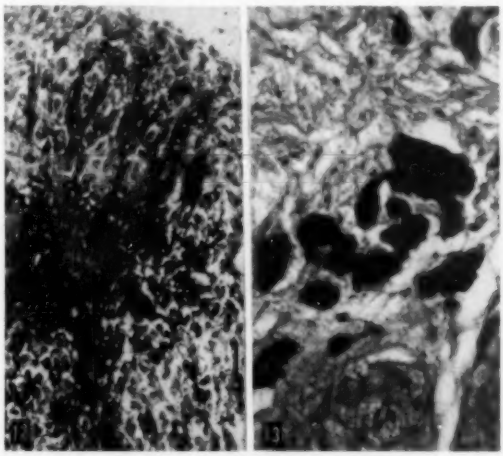
Of the histochemical reactions in the ovarian follicles of the sheep, the only continuously PAS-positive elements are the zona pellucida and the follicular membrane. The reaction in the latter is due to the hyaluronic acid concentration of the basement membrane.

The zona pellucida, however, in addition to its affinity for basic fuchsin even after diastase treatment, also showed a persistently strong metachromasia in every follicle. This reaction is assumed to be due to the presence of acid mucopolysaccharides which, in addition to the protein component, seem to be present in the zona pellucida throughout follicle development.

Although the liquor folliculi and the membrana granulosa cells in small follicles showed only weak affinity toward PAS, the reactions became stronger with the increase in follicular size. In the last stages of development, these components became metachromatic.

As far as the interpretation of the reaction is concerned, Allen and Doisy [2] and Allen [1] showed that the follicular hormone is a mucopolysaccharide. This also is the explanation for the increased strength of the PAS reaction in the atrophying follicles which, according to Mey,[30] Seiferle,[35] and Hofliger,[21] act in the ungulate animals in the same way as the interstitial glands act in the canine species and in rodents.

The presence of lipids in the corpora lutea of different mammals is an established fact and is associated with progesterone secretion. Whether the lipid globules within the membrana granulosa cells preceding rupture can be regarded as an early appearance of a small amount of progesterone is not known.



Sections of Sheep Ovaries

Fig. 12—A newly ruptured follicle showing an alkaline (nonspecific glycero-) phosphatase reaction. x 200.

Fig. 13—An old corpus albicans showing periodic acid-Schiff reaction. x 400.

An interesting feature of the sheep ovary was the acidophilic cytoplasmic components of the large corpus albicans cells. The cytoplasm of the cells was stained strongly with PAS, was weakly metachromatic, and appeared to contain a yellowish lipid which could not be dissolved by fat solvents. Lipid-containing pigments, according to Lubarsch [25] and Ciaccio,[7] can be divided into two groups, *i.e.*, chromolipids, which lose their lipid reactions after sulfuric acid and ammonia treatment, and abnützungspigmente, which do not lose their lipid reactions and are pigments of wear and tear. Since the pigment in these cells is not soluble in ammonia and sulfuric acid it is assumed to belong to the latter group, and the cell is assumed to be a macrophage.

It was found that atresia could occur at any stage of the follicular development in the sheep.

The period which is reported to be most favorable for conditions causing follicular atresia is the second half of pregnancy. In this study on nonpregnant material, the largest numbers of atrophying follicles always were found during metestrus and diestrus. It is presumed that the wave of degeneration is caused either by the discontinuation of follicle-stimulating hormone secretion or by the beginning of luteinizing homone activity. This theory does not provide any explanation for the escape of some large follicles from the atrophic wave; neither does the assumption that the folliculin in the liquor folliculi might act as a protective agent, since many large follicles which, according to this theory, should contain a protecting amount of estrogen are affected by the degenerative wave, while many of the smaller follicles which should contain less estrogen escape.

SUMMARY

1) The morphological and histochemical features of the ovary of the ewe are described.

2) An account is given of the morphological characteristics and the common histochemical reactions involved in oogenesis, follicular development and growth, follicular atresia, corpus luteum formation, and degeneration in the ovaries of 60 nonpregnant ewes.

3) Polyovular follicles and multinuclear oocytes were present during proestrus and estrus. Their number was highest during estrus.

4) The histochemical reactions in the follicle indicate the presence of mucopolysaccharides, phosphatase enzymes, and lipids.

5) The possible connection between the presence of mucopolysaccharides and follicular hormones is discussed.

6) The atresic wave affected the follicles during metestrus and diestrus, irrespective of their size and development.

7) Atresic follicles contained stronger reacting mucopolysaccharides than healthy follicles. The possible significance of the reaction is discussed.

8) The luteal cells were derived from the membrana granulosa and the theca interna.

9) Histochemical reactions in the corpus luteum and in the pigment cells of the corpus albicans are described.

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Histochemical Studies on the Uterus of the Sheep

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THE ALTERATION of lipid and other histochemically demonstrable substances in the uteri of primates and rodents is described many times in the literature. However, there is no information about their presence in the uteri of the seasonally polyestrous animals.

It was thought timely to perform a study on the histochemically demonstrable substances in the uterus of the ewe. In view of the large number of histochemical methods now recognized, this investigation was limited to substances already identified in the uteri of other mammals.

MATERIALS AND METHODS

Specimens from the genital tracts of 60 Scottish Blackface ewes were used. A previous paper described the oviduct secretion in the same animals.[14]

The specimens represented a complete sexual cycle. The important part of the material was obtained from 10 multiparous experimental ewes which were kept on a college pasture with a teaser ram and had shown estrus regularly for at least two consecutive cycles before specimens were obtained. The material from these 10 ewes was recovered by hysterectomy and was put into the required fixatives immediately.

The rest of the material (50 specimens) was obtained at the Glasgow abattoir from freshly slaughtered sheep. The correct phase of the cycle in the abattoir ewes was estimated from the gross anatomical and microscopical appearance of the genital tracts and ovaries, and was compared with those obtained from the experimental animals. These 50 specimens were collected to serve as controls and to supplement the information from the ten experimental specimens. Since all confirmed the results observed on the specimens from the 10 experimental ewes, they were regarded as normal.

In addition to the histochemical fixatives, specimens were fixed in Bouin's fluid[7] and Regaud's solution.[7] For those techniques which did not require freezing, the tissues were embedded in paraffin and sectioned at 6 μ. All these sections were stained routinely with Masson's trichrome stain.[6]

The histochemical techniques performed can be divided into the following groups:

Inorganic Substances—Iron.—Prussian blue and Turnbull's blue for ferric, ferrous, and organic (masked) iron, Lavollay's hydroxyquinoline test[11] for iron, Humphrey's dinitroresorcinol test,[11] and the hematoxylin-lake method (hemosiderin iron)[22] were used.

Organic Substances—Polysaccharides.—The periodic acid-Schiff (PAS) method according to McManus[20, 21] and Hotchkiss[16, 17] was used. Only occasional sections were counterstained with hematoxylin. If staining with PAS was positive, sections were treated (1) with diastase* for 30 minutes at 37 C. to eliminate the presence of glycogen, (2) with 1,000 units of hyaluronidase (Hyalase†) per 100 ccm. of buffered solution for two hours at 37 C.[18] to establish whether the positive reaction is due to the presence of hyaluronic acid, and (3) with warm methanol-chloroform mixture for 12 hours[19] to determine if the positive reaction was caused by the presence of glycolipid. If controls 1, 2, and 3 were negative (if the material continued to react with PAS after the respective treatment), sections were stained (4) with Southgate's mucicarmine, (5) with toluidine blue, and (6) with celestine blue[13] to test for the presence of mucopolysaccharides.

Nucleic Acids.—For the staining of ribonucleic acids, the methyl green-pyronine stain was used. Control specimens were treated with Fisher's mineral acid extraction method.[8]

Lipids.—The Sudan IV stain, according to Herxheimer's method, and hematoxylin, as in the Lorrain-Smith-Dietrich method[6] for the general staining of lipids and lipoids, were used. Baker's acid hematin test[2] and the pyridine extraction method were used to establish the presence of phospholipids, and Schultz's technique was used to determine if the lipid was cholesterol.

Enzymes.—The methods for alkaline and acid phosphatase according to Gömöri[12] were used, with the incubation period alternating between one and two hours. The treatment of controls was identical with that advocated by him.

GENERAL DESCRIPTION OF THE MUCOSA OF THE SHEEP UTERUS

The mucosa of the bicornate uterus of the sheep, like that of other ruminants, consists of regularly interchanging glandular and nonglandular areas (fig. 1). The nonglandular areas, or caruncles, are but-

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* Diastase is produced by the British Drug House, England.

† Hyalase is produced by Benger and Co., England.

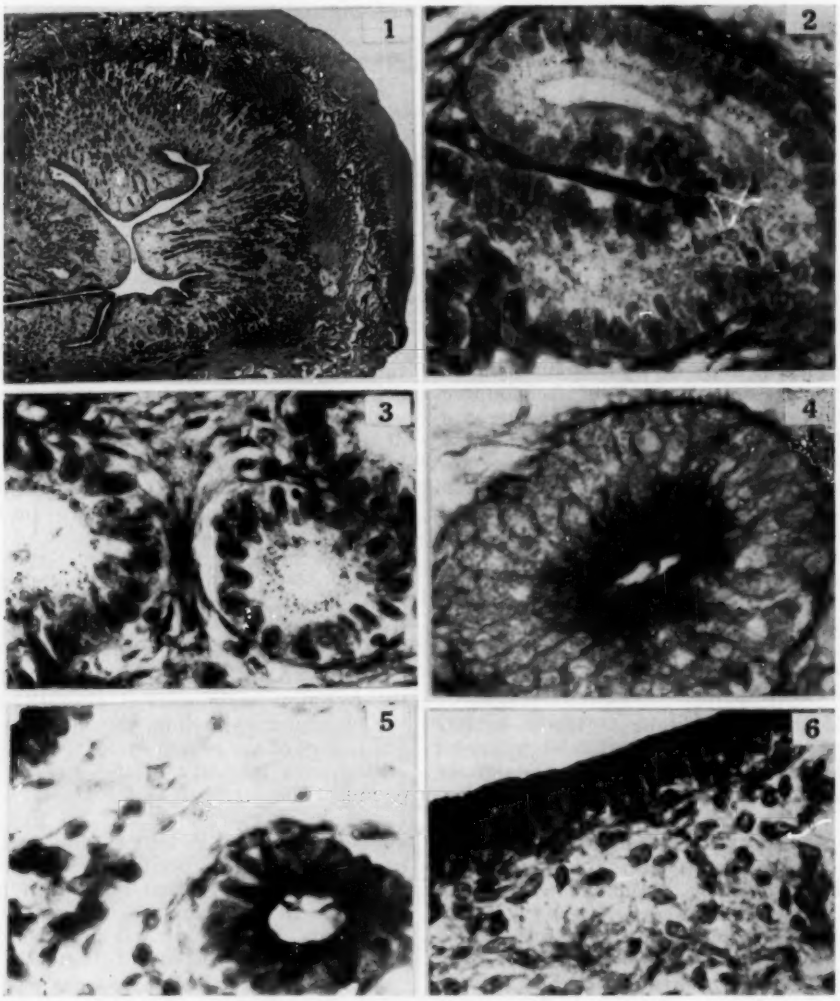

Fig. 1—Cross section of an ovine uterus, showing caruncular and glandular areas. H & E stain; x 15.

Fig. 2—Inorganic iron granules in the uterine gland of an estrous ewe. Turnbull's blue stain, brazilein counterstain; x 400.

Fig. 3—Inorganic iron granules in the uterine gland of a diestrous ewe. Turnbull's blue stain, brazilein counterstain; x 400.

Fig. 4—Uterine glands from a metestrous ewe. Notice the periodic acid-Schiff positive substance in the distal part of the gland. x 400.

Fig. 5—Alkaline phosphatase reaction in the uterine glands of an early diestrous ewe. Notice the strong reaction of the distal surface of glandular cells; the nuclear membranes show weaker reaction. The incubation time was one and one half hours. x 400.

Fig. 6—Alkaline phosphatase reaction in the uterine glands of an early diestrous ewe. Notice strong reaction in the distal part of the epithelial cell. Due to longer incubation (2 hours), the nuclear membrane also reacts more strongly. x 400.

TABLE 1—The Relation of Uterine Secretion to Different Phases of the Sexual Cycle

Secretion	Proestrus	Estrus	Metestrus	Early diestrus	Mid-diestrus	Late diestrus
Iron	++	+++	+++	++	+	—
Mucoprotein	+	+	+++	+++	—	—
Ribonucleic acid	—	+	+	++	—	—
Lipid	—	—	+	++	+++	—
Alkaline phosphatase	—	+	++	+++	—	—

The relative strength of the reactions was evaluated on an empirical basis from the slides observed under the light microscope. — = absence of reaction; + = weak reaction; ++ = moderately strong reaction; +++ = very strong reaction.

ton-like prominences. They are raised above the uterine surface and appear jet black in the majority of Blackface sheep, due to the high melanin content of the mucosa. The number of caruncles has been described to vary between 60 to 120.[13]

The uterine epithelium of the nonpregnant sheep undergoes cyclical alteration during the estrous cycle. It is lowest in late diestrus and early proestrus, increases in height in estrus, and reaches its maximum height in metestrus and early diestrus. A leukocytic migration through the uterine epithelium takes place during the whole sexual cycle, with the highest concentration during diestrus, at the time when the corpus luteum begins to regress.[19]

HISTOCHEMICAL OBSERVATIONS ON THE SURFACE AND GLANDULAR EPITHELIUM

Inorganic Substances—Iron.—In late proestrus, minute iron granules appeared to develop in the centers of the glandular cells. At estrus and metestrus, their number and staining intensity increased and the granules occupied large areas in the distal parts of the cells.

In early diestrus, the number of the iron particles decreased and they were found either in a narrow strip beneath the secretory surface or within the glandular lumen (fig. 2, 3). No iron particle was present in late diestrus or anestrus. No masked iron was observed in the uterus of the sheep.

Organic Substances—Polysaccharides.—No PAS-positive reaction was observed in anestrous or late diestrous specimens. In the proestrous and estrous uteri, however, there was a narrow layer of PAS-positive material in the distal parts of the surface and glandular epithelium cells. During metestrus and early diestrus, the PAS-positive area increased in depth in both surface and glandular epithelial cells (fig. 4). The increase was greater in the glandular cells, and some of this apparently secretory material appeared in the glandular lumen.

The strength of the PAS reaction did not change after treatment with diastase or hyaluronidase, indicating that the secretion was neither glycogen nor hyaluronic acid; it remained active after incubation with methanol-chloroform solution, a proof of the absence of glycolipids. Celestine blue and toluidine blue failed to cause a metachromatic reaction and Southgate's mucicarmine did not stain it, proving that the material was not an acid mucopolysaccharide. Since the secretory material was not glycogen, hyaluronic acid, or an acid mucopolysaccharide and still gave the positive PAS reaction, it is regarded as mucoprotein.

Nucleic Acids.—While the strength of the pyronine stain showed alterations in the glandular epithelium during the various phases of the sexual cycle, it remained of the same intensity in the surface epithelium. The strength of the reaction was lowest in proestrus, showed an increase during estrus and metestrus, and reached its maximum at the beginning of diestrus. It then remained at that level until middiestrus. In both surface and glandular epithelium, the staining was negative during anestrus and the end of diestrus.

Lipids.—In uteri of metestrous ewes, a number of minute lipid globules were present in the proximal part of the surface and glandular epithelial cells. The number of globules increased during early diestrus and occupied a larger part of the cell than during the earlier part of the cycle. At middiestrus the granules spread to the distal parts of the cells and also appeared in the glandular lumen. The globules always were small (fractions of 1 μ in sheep), and there never was any coalescence visible between them. The globules were well stained with all the lipid dyes enumerated in the section dealing with techniques, with the exception of cholesterol. Pyridine

completely extracted the lipid. No lipid was stained in the paraffin-embedded sections.

Phosphatase Enzymes.—Nonspecific cytoplasmic alkaline phosphatase reaction was observed in the glandular epithelium, as well as in the surface epithelium, but only the distal parts of the cells gave the reaction. The strength of the alkaline phosphatase reaction changed during different phases of the cycle. It was lowest during proestrus and increased markedly during estrus and metestrus. The reaction was maximum during early diestrus (fig. 5, 6), and no reacting material was observed during anestrus.

The acid phosphatase reactions were not consistent enough to enable any consideration of results to be made.

All the reactions, with the exception of the lipid globules, showed differences of strength in the various segments of the same gland; *i.e.*, if the proximal part of the gland was reacting strongly, then the reaction in the distal part was weaker, and if the distal part showed vigorous reaction, then the reaction in the proximal part was less.

DISCUSSION

The histochemically reacting substances in the ovine uterus, with regard to the time of their appearance within the sexual cycle, can be divided into two distinct groups: (1) those which appear in proestrus, show an increase in amount or strength of reaction during estrus, and disappear thereafter, and (2) those which first appear only after estrus, increase in amount during diestrus, and disappear later.

It is evident that inorganic iron belongs to the first group, lipid is characteristic of the second, while mucoprotein, alkaline phosphatase, and ribonucleic acid occupy a place between them.

The inorganic iron secretion showed considerable alteration in different phases of the sexual cycle. Since the granules have appeared only in late proestrus and have disappeared after metestrus, they are assumed to be under the influence of follicular secretion. Up to now no author has mentioned presence of inorganic iron in the uterine milk of the pregnant sheep, although inorganic iron is known to be present in the uterine milk of the early pregnant horse.[2] When discussing the presence of inorganic iron, it might be supposed that it has originated from estrual bleeding during estrus. In this breed of sheep, however, the author has never observed any bleeding—only comparatively mild hyperemia. Furthermore, the inorganic iron granules already are visible in proestrus, whereas there is no uterine hyperemia at this phase of the sexual cycle, and the granules appear to move within the cells from the proximal to the distal borders. Therefore, its origin as a secretion, until proved otherwise, is regarded as a valid working hypothesis.

Reynolds found glycogen in the human uterus,[23] while the PAS-positive material in the ovine uterus proved to be mucoprotein. Like the inorganic iron granules, the PAS-positive material appeared in proestrus, but the maximum strength of the reaction developed only in diestrus, it being more like the lipid granules in this respect. Consequently, it is thought that the uterine epithelial mucoprotein secretion is influenced by both the folliculin and the progesterone secretion. It is likely that, in addition to cellular debris, this mucoprotein secretion is the cause of the high protein content of the uterine milk in the early pregnant sheep. The latter can amount to approximately 9.9 per cent.[2]

Bremer *et al.*[5] found alterations in the ribonucleic acid content of the glandular cells of the human endometrium in different phases of the sexual cycle, while the content in the nonglandular epithelium remained unchanged during the whole sexual cycle. While they found the highest ribonucleic acid level in the human endometrium to occur in the preovulatory phase, the ribonucleic acid reaction in the uterus of the sheep reached its peak during early diestrus. In the sheep, this level of the reaction was maintained until the middle of diestrus; a rapid decrease of the ribonucleic acid took place thereafter. The significant difference between the two findings is this: In the human endometrium, the maximum amount of ribonucleic acid is present at the time of the highest follicular activity; in the domestic sheep, it is present when the progesterone activity is highest.

Lipid showed periodical alteration. It was absent in proestrus and estrus and reappeared after metestrus, reaching its maximum in diestrus. The timing of the

appearance of the lipid suggests that estrogens suppress or eliminate epithelial fat, while progesterone causes its reappearance. The same relationship was shown to exist in the uteri of human beings,[10] rabbits,[9] and rats.[1]

As far as the nature of the lipid is concerned, it appears that it was not glycolipid, since it did not react with PAS. Although the Schultz cholesterol test[12] remained negative, it is possible that the amount present was too low to react, considering the extreme nature of the reaction. Despite the negative reaction, the presence of cholesterol can not be ruled out. However, pyridine suppressed the positive acid hematin reaction, indicating that the lipid had a strong phospholipid component.[3]

The strength of the reaction of the nonspecific cytoplasmic alkaline phosphatase enzyme appeared to follow the changes of the ribonucleic acid secretion. Therefore, it appeared to be waning in the follicular phase and waxing in the luteal phase. The presence of the phosphatase enzyme during early diestrus is regarded as the sign of the high metabolic activity within the uterine epithelial cells in this phase of the cycle.

SUMMARY

1) Inorganic iron, mucoprotein, ribonucleic acid, lipid, and alkaline phosphatase were found in the uterine epithelium.

2) All these substances showed cyclical alterations.

3) The changes in the amount of inorganic iron indicated that it is influenced by the folliculin, while the lipid and mucoprotein were dependent on the progesterone secretion of the ovary.

4) Ribonucleic acid and alkaline phosphatase appeared at the time of high progesterone activity.

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The Persistence of a Genital Vibrio Infection in a Herd of Sheep After an Outbreak of Vibrionic Abortion

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IT HAS BEEN INDICATED by several investigators [1, 2, 9, 10] that vibrionic abortion occurs only in one lambing season in the same herd. No description of ovine Vibrio abortion occurring in two or more succeeding years has been found in the literature.

This is an account of an investigation in a herd of sheep during a period of one and one half years after the occurrence of spontaneous vibrionic abortions.

MATERIALS AND METHODS

The 180 sheep at the State Veterinary Experimental Farm for Sheep, where the authors previously reported an investigation during an outbreak of spontaneous vibrionic abortion in the spring of 1956, were used. Vibrios were found in several sheep showing no clinical signs of vibriosis, as well as in the aborted fetuses. Biochemical testing, serological examination, and inoculations in pregnant guinea pigs seemed to show that all the isolated vibrios belonged to the same strain.[4]

Investigation in the herd was continued in the following spring and in the fall, one and one half years after the abortions occurred. The material for investigation was taken from the vaginal mucus of 151 sheep, the fetal fluid at 28 normal parturitions, the gastric contents from 15 newborn, healthy lambs, and the semen from 6 rams.

The collection and inoculation of the material and the serological examinations of the Vibrio strains isolated were carried out as described by Eide and Helle.[4]

Vibrios were isolated by the method of the Pasteur pipette described by Florent.[5]

Pure cultures of the vibrios isolated were tested biochemically as described by Bryner and Frank.[3]

Pregnant guinea pigs were inoculated with the Vibrio by the method first introduced by Garm [7] and later used in the routine diagnostic work at this department.

RESULTS

The Clinical Course.—There have been no clinical signs of vibriosis in the herd during the one and one half years of investigation since the abortions. During this period, all of the rams used, including several of the infected rams, showed normal fertility.

Investigation One Year After Abortions.—During the lambing season one year after the abortions, samples of vaginal mucus were taken from 66 sheep. Vibrios were found in 16 samples, four of which were from virgin yearlings (gimmers). From seven of these positive samples, the vibrios were isolated in pure cultures.

One of the samples from the fetal fluid taken at 28 normal parturitions was positive, but the attempt to isolate the vibrios in pure culture was not successful.

Of the samples of gastric content taken from 15 healthy newborn lambs, one was positive, and vibrios were isolated in pure culture.

Investigation One and One Half Years After the Abortions.—Samples were taken of vaginal mucus from 59 ewes and from

TABLE 1—Biochemical Tests of Vibrios Isolated from Sheep

When isolated	Material	Strain	Catalase	H_2S	NO_2	Deep stab
Spring, 1 year after the abortions	Vaginal mucus from pregnant ewes *	104–53	0 mm.	–	+	±
		170–54	2 mm.	–	+	±
		41–53	0 mm.	–	+	±
		58–54	0 mm.	–	+	±
	Vaginal mucus from gimmers	22–56	0 mm.	–	+	±
		58–56	0 mm.	–	+	±
		35–56	0 mm.	–	+	±
	Gastric content from normal lamb	205–50	0 mm.	–	+	±
Fall, 1.5 years after the abortions	Vaginal mucus	24–57	3 mm.	–	+	±
		53–51	0 mm.	–	+	±

* None of these ewes had ever aborted.

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TABLE 2—Agglutination Tests on Vibrio Strains Compared with Strains Isolated at the Time of Abortions in the Same Sheep Herd

O + H antigen	Time of abortion		Spring, 1 year after abortion								Fall, 1.5 years after abortion	
	Aborted fetus	Vaginal mucus, pregnant ewe	Vaginal mucus, pregnant ewes				Vaginal mucus, gimmers			Gastric content, normal lamb	Vaginal mucus	
O + H antiserum	149–53	32–53	104–53	170–54	41–53	58–54	22–56	58–56	35–56	105–50	24–57	53–51
Aborted fetus 149–53	5120*	80	5120	5120	0	0	5120	5120	5120	40	80	160
Vaginal mucus, pregnant ewe 32–53	5120	5120	5120	5120	160	160	0	5120	5120	160	640	5120

* The figures correspond to the highest dilution giving complete agglutination.

26 lambs, 6 months old, before mating. Samples from 3 ewes and 2 lambs were positive, and vibrios were isolated in pure cultures from two of the samples.

Vibrios were not demonstrated in the semen from 6 rams, although several of the rams had been infected previously.

Biochemical Examination of the Vibrio Strains Isolated.—The results of the biochemical examination of ten strains are shown (table 1). Two of the strains showed slight catalase activity (2 and 3 mm.), and the others were negative. No strains showed H_2S production, while the nitrate reduction was positive. In stab culture, it was possible to observe growth at the top of the medium and slight growth along the stab.

Serological Examination of the Vibrio Strains Isolated.—Antiserum (O + H) was prepared from two Vibrio strains isolated, at the time of the abortions, from an aborted fetus and from the vaginal mucus from a pregnant ewe. The prepared serums showed titers of 1:5,120. These were titrated with antigen prepared from the isolated Vibrio strains (table 2). The agglutination tests showed titers indicating an antigen relationship with Vibrio strains isolated one and one half years earlier.

Inoculation of Pregnant Guinea Pigs.—The results of these inoculations are shown (table 3). Altogether, of the 20 guinea pigs inoculated with ten strains, 7 aborted, but vibrios were isolated from the aborted fetuses of only 5.

DISCUSSION

Since the abortions started, the herd has been kept under close control. Examination has shown that several animals carried vibrios for as long as 18 months after abortions occurred, without clinical signs of vibriosis in the herd.

Most of the Vibrio strains isolated at the time of the abortions showed catalase

TABLE 3—The Abortifacient Effect on Pregnant Guinea Pigs of Vibrio Strains Isolated from Sheep

When isolated	Material	Strain	No. guinea pigs injected	No. abortions	No. confirmed Vibrio abortions
Spring, 1 year after the abortions	Vaginal mucus from pregnant ewes *	104–53	2	2	1
		170–54	2	1	1
		41–53	2	0	0
		58–24	2	0	0
	Vaginal mucus from gimmers	22–56	2	0	0
		58–56	2	1	0
		35–56	2	1	1
	Gastric content from normal lamb	205–50	2	1	1
Fall, 1.5 years after the abortions	Vaginal mucus	24–57	2	0	0
		53–51	2	1	1
	Control group	Sterile medium	4	0	0

* None of these ewes had ever aborted.

activity, although not more than 5 mm. In stab culture tests, these strains showed only growth on the top.[4] Among the Vibrio strains isolated one and one and a half years later, only two showed catalase activity (2 and 3 mm.) and, in stab cultures, all the strains showed slight growth along the stab, besides growth on the top. Otherwise, the results of biochemical tests were identical. Serological examinations showed a relationship between the strains isolated during and up to one and one half years after the abortions occurred.

During an earlier investigation, ten of the strains isolated at the time of abortion were inoculated into 19 guinea pigs, 13 of which aborted; vibrios were found in the aborted fetuses from 10 of the 13.[4] However, the last vibrios isolated had less abortifacient effect on pregnant guinea pigs, since only 7 of 20 aborted and vibrios were isolated from only 5 of the 7.

Immunity possibly may be a factor in the absence of clinical signs in the infected sheep. It must be expected that the young animals are orally infected long before sexual maturity [6] or by infection at parturition.[4] It often is difficult to find the source of infection leading to spontaneous vibrionic abortions in a herd of sheep. It is possible that a herd such as this one may be the source of infection of other herds more susceptible to the disease.

Summary and Conclusions

1) Systematic investigation has been carried out in a herd of sheep for one and one half years after an outbreak of spontaneous Vibrio abortion. Vibrios have been isolated from vaginal mucus and from material taken at normal parturitions. During this period there have been no clinical signs of genital vibriosis.

2) The investigation seems to show that the vibrios isolated at the time of the abortions and those isolated during the period of one and one half years afterward belong to the same strain; however, the pathogenicity is lower in the last isolated vibrios.

3) The investigation shows that animals in the herd may carry vibrios long after abortions cease, even when there are no clinical signs of infection in the herd.

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Chronic Copper Toxicosis in Sheep

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THE EMERGENCE of copper as an important feed additive and the use of copper salts as anthelmintics in various species have stimulated a new investigation into the toxic effects of these compounds in sheep, an animal known to show marked clinical response to repeated doses of copper.

A substantial review of the literature related to the toxicity of copper for sheep was published by Boughton and Hardy, in 1934.[1] With this review, they reported the results of their own investigations, in which they demonstrated that copper sulfate (2 Gm./day) was fatal in 25 to 42 days, giving a typical picture of hemolytic jaundice and sudden death. Intermittent dosing with copper sulfate for periods exceeding 12 months gave no untoward effects.

In more recent years, a review of the biological activity and toxicity of copper has been published by Marston.[5] In this work, he pointed out that sheep, like other ruminants, accumulate copper in the liver until high levels are obtained but that, unlike other animals, the copper then "apparently spills out suddenly,"[2] causing the hemolytic crisis.

Other reports concerning clinical observations of the toxicity of copper have appeared in the veterinary literature and studies have been made on the effect of dietary molybdenum on the accumulation of copper by the liver.[3, 4, 7]

This investigation was designed to incorporate in one report detailed information on the gross and microscopic pathological changes and liver and blood copper values following daily administration of a fixed quantity of copper. Since it has been reported that blood copper levels are of uncertain value in diagnosing chronic copper poisoning in cattle,[4] determinations of blood copper were made throughout these experiments to determine the time of rise in relation to the health of the animal.

MATERIALS AND METHODS

Four farm-reared sheep, 8 months old, were placed in a cement-floored pen, approximately 20 by 20 ft. The ration consisted of prairie hay, alfalfa hay, and a grain mixture (0.33% oats, 0.33% barley, 0.33% cracked corn). Salt and water were permitted ad libitum.

The copper used in these experiments was supplied as cuprous iodide, technical grade.*

The sheep were confined to the pen, except during weekly weighings. Cuprous iodide (750 mg./animal/day), contained in a hard gelatin capsule, was administered individually. Blood samples were taken weekly from the jugular veins. Necropsies were conducted as soon as possible after death, and tissues for histological study were sent to an independent pathologist who was uninformed as to the nature of the test.

Copper determinations from the blood and liver were performed according to the method of Peterson and Bollier,[6] in which the color developed in the deproteinized blood or diluted tissue by the addition of bicyclo-hexanone oxaldihydrazone (Cuprizone†) at a pH of 9 is determined spectrophotometrically at 600 mμ.

RESULTS

The values for the weekly body weights of the animals, the serum copper values, and the postmortem liver copper values are shown (tables 1, 2, 3).

Chronology.—Twelve days after the experiment started, all the sheep had slight serous nasal discharges of approximately 24 hours' duration. On the twenty-first day, sheep 1 developed a small hygroma on the left carpal joint, but this disappeared without treatment.

On day 67, sheep 3 was weak, with a dull appearance. Respiration was rapid, giving the animal a decided rocking movement. Closer examination showed injected, muddy-looking mucous membranes and sclera. The remaining ocular tissues were jaundiced. The skin was yellow, especially on the inner surface of the ears. The animal died during the night. Necropsy, the following morning, revealed jaundiced subcutaneous tissues, omentum, and mesentery. The liver was dark. The kidneys were remarkably similar to those in "pulpy kidney disease." The bladder contained bloody urine of a port wine color. No tissues were taken for microscopic study, since decomposition had started.

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* Supplied by the Morton Salt Co., Chicago, Ill.

† Cuprizone was supplied by the G. Frederick Smith Chemical Co., Columbus, Ohio.

TABLE 1—Body Weights of Sheep (lb.)

Sheep (No.)	Control	Experiment weeks 1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	61.6	62.7	66.0	67.0	71.5	74.8	73.7	79.2	72.6	75.8	70.4	71.9	.....*	.....	.....
2	70.4	69.3	78.1	83.0	87.0	91.7	92.8	94.6	98.2	100.2	91.3	91.0	87.7	82.5	78.1†
3	68.7	69.7	73.7	74.8	83.5	83.6	84.0	84.7	84.7	84.7	.....**	.....	.....	.....	.....
4	79.2	78.1	84.7	90.2	92.7	96.8	95.7	99.0	99.0	102.3	91.3	.....‡	.....	.....	.....

* Died July 29, 1957; † died Aug. 23, 1957; ** died July 15, 1957; ‡ died July 22, 1957.

On day 74, sheep 4 was observed to be slower and weaker than the remaining 2 and to be voiding coffee-colored urine. The mucous membranes, skin, and ocular tissues were jaundiced. The animal died the same day and was necropsied immediately. Jaundice extended throughout the subcutaneous tissues, fat, omentum, and mesentery. The liver was enlarged and dark yellow. The gallbladder was distended. The spleen was enlarged and considerably darker than normal. The kidneys were enlarged, dark, and hemorrhagic, and the lining of the renal hilus was jaundiced. The bladder was filled with coffee-colored urine. The heart had epicardial and endocardial hemorrhages at the upper part of the ventricles. The lungs were atelectic along the borders and there appeared to be a small amount of "red hepatization" in the right lobe.

Sections of liver, kidney, and spleen were preserved in 4 per cent formaldehyde and submitted for histological examination. The report of the pathologist was as follows:

Gross Pathology.—The piece of liver showed a surface of dark red brown throughout with speckled areas of a greenish gray nature. The speckling was quite uniform and striking. The piece of tissue was rather soft to gum rubber consistency. Cross section revealed rather extensive areas of light gray green tissue in which were scattered areas of dark red. The piece of spleen revealed the capsule to be gray, thickened, and dull with projecting brownish areas. Cross section revealed a rather uniform dark red brown through which increased trabeculations appear. No malpighian bodies were noted. The remaining tissue (kidney) was of gum rubber consistency and a dark gray color. Cross section revealed this gray area to extend almost entirely through the mass.

Histopathology.—Sections through the kidney showed pigmented casts in the tubules. The spleen showed diffuse pulpy hyperemia. The liver showed central degeneration, ischemia, and hemorrhage with widespread early necrosis of parenchymal cells in some areas about the central veins and heavily pigmented areas as well.

Modified Levaditi stains by the Nieto method were applied for spirochetes but none were found. Specific iron stains revealed that the pigment in all of the organs was blood pigment rather than bile pigment.

Diagnosis.—Acute hemolytic disease in sheep with extensive blood pigmentation of liver and spleen. Pigment casts in the tubules, and ischemic type central degeneration of the liver with considerable necrosis of the central type.

TABLE 2—Blood Serum Copper Values (μg./100 ml.)

Sheep (No.)	Experiment weeks 1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	102	82	98	95	108	94	124	169	172	256	253	.....	.....	.....
2	191	124	86	192	152	121	186	102	94	244	207	.....	218	195
3	111	89	73	122	162	157	170	142	170	.....	.....	.....	.....	.....
4	111	96	77	133	113	110	126	102	113	253	.....	.....	.....	.....

TABLE 3—Liver Copper Content of Sheep at Necropsy

Sheep (No.)	Wet basis (p.p.m.)	Dry basis (p.p.m.)
1	750	2,500
2	649	2,160
3	474	1,580
4	596	1,985

On day 77, sheep 1 developed slight jaundice in the nictitating membranes and died on day 81. The gross necropsy findings were identical to those from sheep 3, and histopathological findings differed only in that evidence of bile staining was found in the liver, with accompanying inflammatory changes.

Sheep 2, the only male in the test, died on day 106. He showed no hemolytic signs and no jaundice. Histological study revealed only marked edema of the lungs and a high-grade, passive hyperemia of the liver.

DISCUSSION

The gross signs and necropsy findings presented here agree with those previously reported, except those for sheep 2. There is no apparent explanation for the fact that this animal, although developing both liver and blood copper content to the same extent as the female animals, failed to show signs of hemolytic jaundice. Although one could postulate a greater resistance to these changes in the ram, it is felt that this finding is indicative of the fact that hemolysis and jaundice are not necessary criteria for a diagnosis of copper poisoning.

As shown (table 3), the liver copper content is indicative of poisoning from this metal. In contradistinction to the report of Cunningham,[2] demonstrating blood copper values to be of little value in the diagnosis of poisoning in cattle, evidence is presented (table 2) to show that, in sheep, constant administration of 250 mg. per day of cuprous ion causes a definite and early rise in the blood copper levels. In 3 of the 4 sheep, these blood copper levels reached values of 244 μg./100 ml. or higher before death of the animal.

The facts that the copper content of the blood does increase with continuous administration of cuprous ion and that the peak levels were attained five to 35 days before death of the animal are interpreted as indicating that the sudden rise in blood copper does not represent a sudden outpouring of copper from the liver as reported by Marston,[5] but rather that the liver capacity for removing copper from the blood has been exceeded and that further absorption of copper is reflected in a sharp increase in blood copper levels. Death then occurs from acute liver failure with secondary jaundice.

SUMMARY

The clinical changes, gross pathological findings, histopathological findings, and blood and liver copper levels following daily administration of 250 mg. per day of copper to sheep are presented. Interpretation of the data leads to the conclusions that the blood copper levels in sheep may be used for a definitive diagnosis of poisoning by this ion and that the hemolytic syndrome commonly associated with copper poisoning is not an essential criterion for its diagnosis.

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Effect of Stable Iodine on Uptake of Radioiodine in Sheep

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THIS PAPER reports the results of two studies designed to evaluate the depression in thyroid uptake of I^{131} that occurs in the presence of approximately normal intake of I^{127}, as compared with the uptake of I^{131} in the presence of marginally goitrogenic amounts of stable iodine. The studies described were performed in support of a long-term study on the biological effects of I^{131} in sheep fed a ration low in stable iodine.[1] Stable iodine content of the ration was limited to 0.3 to 0.5 mg. per day to simulate range conditions of areas where herbage is considered marginally goitrogenic[3] and to insure maximum thyroid uptake of I^{131}.

Information of this type, although of a preliminary nature, is essential to (1) the application of data obtained in experiments conducted under marginally goitrogenic conditions, (2) the analysis of observations on sheep fed a ration containing recommended amounts of stable iodine, and (3) the estimation of the supplemental feedings of iodine required to minimize the biological hazard from I^{131} in case of serious contamination.

MATERIALS AND METHODS

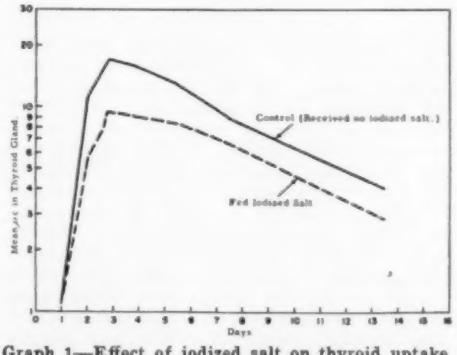
In the first study, 22 yearling Suffolk sheep (mean body weight, 56 kg.) were given a 30 microcurie (μc.) tracer dose of I^{131}. Of this group, 15 then were placed on a feeding regimen which included ½ lb. of grain mixture containing 0.25 oz. of iodized salt. This method of administering iodized salt is used commonly by many farmers in this area. The average daily intake under this regimen was about 1.1 mg. of I^{127} per day. When external monitoring of the thyroid demonstrated that 1 μc. or less remained from the first dose, a second 30-μc. tracer dose of radioiodine was administered.[2] The 7 control animals were not given iodized salt and had not been given any during their lifetimes as members of a control group. Of the 15 animals fed iodized salt, 7 were from this same control group, while the remaining 8 were obtained from a typical farm flock of sheep. The average thyroid weight of the animals in the former group was 7.5 Gm., as compared with 4.2 Gm. for the animals from the farm flock.

The second study involved 15 Suffolk ewes (mean body weight, 72 kg.) which had been maintained for their entire lives on rations low in stable iodine; 8 were given 5.0 μc. and 7 were given 0.15 μc. of I^{131} per day. Four animals from each of these groups were given 2.5 mg. of I^{127} per day, orally, as KI. Thyroid monitoring was performed one or two times daily during the first week and two to three times a week thereafter.

RESULTS AND DISCUSSION

Following the initial tracer dose of the first study, thyroidal uptake of I^{131} by the experimental group was 85 per cent of the level reached by the control group. However, after the experimental group had been fed iodized salt for 18 days, the mean amount of radioiodine in the thyroid glands at 40 hours after a second tracer dose was only about 50 per cent of that in the control group and was independent of the source of the animals and of their thyroid weights (graph 1).

In the second study (graphs 2A, 2B), when the ewes were fed 2.5 mg. of I^{127} daily, the thyroidal content of I^{131}, as determined by external monitoring, was re-



Graph 1—Effect of iodized salt on thyroid uptake in sheep fed 30-μc. tracer doses of I^{131}.

From Biology Operation, Hanford Laboratories, General Electric Co., Richland, Wash.

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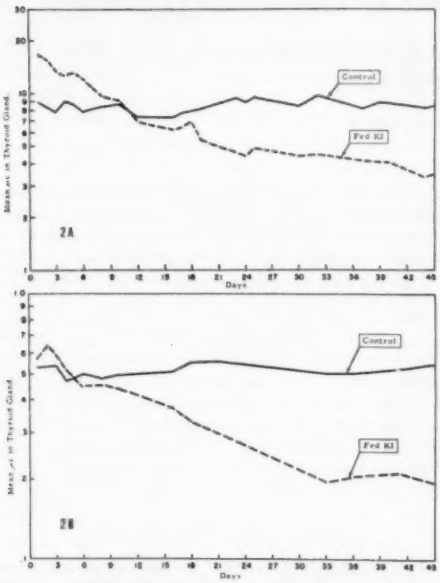

Graph 2A—Effect of daily supplement of KI on thyroidal I^{131} in sheep fed 5.0 μc. of I^{131} per day.

Graph 2B—Effect of daily supplement of KI on thyroidal I^{131} in sheep fed 0.15 μc. of I^{131} per day.

duced in 30 days to less than one half that of the controls. The response to KI supplementation in the group fed 5.0 μc. of I^{131} per day was somewhat less than in those animals fed 0.15 μc. per day, probably due to a slight thyroid damage resulting from the daily doses of radioiodine administered during their lifetimes.[1, 4]

SUMMARY

Supplying iodized salt (in amounts approximating those used by many farmers) to sheep on a ration low in stable iodine reduced thyroid uptake of a single tracer dose of I^{131} by over 30 per cent. Inorganic iodine (KI) administered daily at levels of 2.5 mg. to sheep, maintained on 5.0 and 0.15 μc. of I^{131} daily, reduced thyroid uptake to less than 50 per cent of that of the controls.

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Adrenal Cortical Adenomata in the Goat

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A HIGH INCIDENCE of adrenal cortical adenomata in castrated male goats is noteworthy when compared to the negligible incidence of the same tumors in normal males. These observations, made during the necropsy examination of 831 adult goats, are even more significant in view of the experimental findings on the production of adrenal tumors in small laboratory animals. This discussion presents the gross and microscopic picture of these tumors as seen in the goat, in addition to a report of their incidence as related to sex and castration. It is hoped that it may add to the understanding of the pathogenesis of these tumors in other species, as well as in the goat.

REVIEW OF LITERATURE

Tumors of the adrenal cortex are not uncommon in most species of animals. While ruminants seem to be most frequently affected, reports of adrenal tumors of the goat are almost nonexistent. Richter [11] described a tubular adenoma of the goat adrenal and briefly mentioned a cortical adenoma in one of his case reports. The lack of reports of adrenal tumors or any neoplasia in the goat probably is due to the fact that the goat is not subjected to detailed necropsy examination as frequently as those ruminants of greater economic importance.

Feldman [4] expressed a similar opinion in connection with his observation that tumors are less frequently seen in goats than in some other species. In his summary of ovine neoplasms,[5] he stated that the adrenal glands of this species seem to possess a greater predilection for the development of tumors than any other specific tissues or organs. He reported 10 cases of adrenal tumors in sheep; 7 unilateral and 3 bilateral. These ranged in weight from 15 to 120 Gm., with six of the unilateral and all of the bilateral tumors being carcinomatous and all of them apparently derived from cortical tissue.

Davis *et al.*[2] reported 13 adrenal epithelial tumors in a group of 90 neoplasms of bovine origin and ten adrenal epithelial tumors in a group of 34 ovine tumors. They concluded that the adrenal glands of cattle and sheep have a greater tendency toward cancerous involvement than other internal organs. Adrenal neoplasia accounted for only one of 26 tumors of swine in their study.

Monlux *et al.*[10] reported adrenal cortical tumors of cattle and sheep. This neoplasm accounted for 23 of 186 tumors in cattle, eye tumors being excluded from this number, and five of 14 tumors of sheep. Only four of the bovine tumors were under 5 cm. in diameter and all of the ovine tumors were over 4 cm. in diameter. The bovine cases included 2 steers and 21 females; the ovine cases included 1 lamb and 4 old ewes.

Jackson [8] described tumors of the goat but none of these were adrenal in origin. His series from other species included eight adrenal cortical tumors, of which three were from cattle, three from horses, and two from sheep.

Smith and Jones [13] compiled information on 7,052 neoplasms from five different sources, including Jackson's collection. This series included 16 adrenal cortical tumors in the dog, two in the horse, 34 in cattle, and 17 in sheep; a total of 69 neoplasms. It can be seen that most of the reported adrenal tumors are found in ruminants, but none of these reports gives any significant information as to the relationship of sexual activity.

There is considerable information available on the relationship of gonadectomy to the development of adrenal cortical tumors in laboratory mice. In a review of some of this work up to 1949, Woolley [15] stated that a high percentage of certain strains of male and female mice develop adrenal tumors after gonadectomy. Similar observations were made for mice by Gardner,[6] for hamsters by Keyes [9] and Woolley,[14] and for rats by Houssay *et al.*[7]

MATERIALS AND METHODS

The neoplasms described in this report were collected during the routine necropsies of 831 goats, consisting of 449 dairy goats of mixed breeding from the eastern United States and 382 Angora goats from Texas. Almost all were adults, over 4.5 years old, as indicated by the stage of dentition. There were 188 males, 208 females, and 435 castrated males; the numbers of each breed as compared to their sex are shown (table 1). All were considered normal, healthy animals, as determined by brief observation before death, and there was no clinical evidence of endocrine disturbances.

During necropsy, both adrenals were removed, weighed, and fixed in 10 per cent formalin solution. The glands were incised after fixation and

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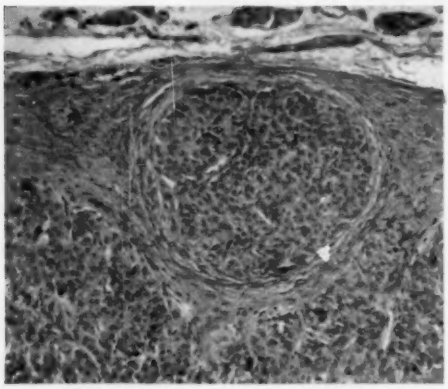

Fig. 1—The nodule of zona glomerulosa in the adrenal capsule is considered normal in the goat, and is not to be confused with cortical adenomata. G1260, H & E stain; x 104.

a small block of tissue was removed for sectioning. The right adrenal usually was cut transversely and the left longitudinally, the left being longer than the right. Single adrenals were incised and sectioned in some of the earlier cases.

In this study, both adrenal glands from 456 goats and single glands from 375 goats were examined microscopically, a total of 1,287 adrenals from 831 goats. The tissue was embedded in paraffin and two or more sections from each block were cut at 6 μ for routine hematoxylin and eosin staining. Thus, only a small area of each gland was examined and many small neoplastic foci may have been missed. A few frozen sections were cut and stained with oil red O for determination of lipid content and distribution.

Some confusion exists as to the classification of adrenal cortical cellular changes in other species, and some investigators might describe a few cases of this series as nodular hyperplasia. There being no previous reports dealing specifically with this neoplasm in the goat, some criteria were set down for the inclusion of a lesion in this series. They agree with the fundamental definition of neoplasia as given by standard textbooks of pathology.[1, 12, 13] Numerous nodular growths of cortical cells are seen in goat adrenals, but these are not described as neoplasia unless the following conditions are met: There must be a definite focal increase in number of cortical cells. These may or may not be contained in connective tissue capsules. The cells of the lesion must fall outside the normal limits of variation of cortical cells as related to size, shape, and staining characteristics of the cell and its nucleus. These cells must grow in a pattern or arrangement not characteristic of the cortex as seen in the normal goat adrenal. In other words, all proliferative lesions derived from adrenal cortex which contain abnormal cells growing in an unusual pattern are included as adenomata. This excludes isolated proliferative nodules of cortex which consist of normal cells in a normal pattern. Also excluded are the numerous capsular nodules of zona glomerulosa cells which Elias[8] described as normal growth patterns for the goat (fig. 1).

GENERAL DESCRIPTION

The majority of the tumors were so small that they were not recognized until the adrenal was examined microscopically. The larger lesions were recognized on the cut surfaces of fixed glands as oval or round areas, slightly paler than the surrounding cortex. Many such pale areas did not turn out to be neoplasms when the slides were examined, and the only positive determination was microscopic examination. Most of these lesions were located in the outer cortex or just beneath the capsule, although a few were associated with bits of cortex folded into the center of the gland. The smaller lesions did not alter the normal thickness of the cortex; larger ones occupied the total thickness of the cortex and projected into the medulla. The capsule rarely bulged outward because of the presence of a neoplasm, but a disruption of internal structure usually occurred. The largest lesions occupied one end of a gland or made up the bulk of a gland. In these cases, the external appear-

TABLE 1—Incidence of Adrenal Cortical Adenomata as Related to Sex and Breed

Breed and sex	Animals with single adrenals examined	Animals with two adrenals examined	Total adrenals	Adrenals with tumors	Total goats examined	Animals with tumors
Angora, male	5	9	23	0	14	0
Dairy, male	127	47	221	0	174	0
Total males	132	56	244	0	188	0
Angora, female	2	2	6	0	4	0
Dairy, female	141	63	267	2	204	2
Total females	143	65	273	2	208	2
Angora, castrated male	62	302	666	157	364	102
Dairy, castrated male	38	33	104	12	71	9
Total castrated males	100	335	770	169	435	111

ance of the gland was altered, with one enlarged end, or the whole structure was oval, rather than the usually slightly elongated shape.

The adenomata described in this report are grouped according to their size on the stained section. Because of the shrinkage occurring in the preparation of a section, these are not true indications of the original size, but they do indicate relative sizes and give a general picture of the original measurement. In cases of multiple lesions, the largest focus was measured. Of 113 goats involved, 55 had adenomata under 2 mm. in diameter, 36 had lesions ranging from 2 to 5 mm., and 22 contained lesions over 5 mm. in diameter, the largest of the series being 11 mm. Another was 10 mm. and two more were 9 mm. in diameter.

In 50 goats, the lesion was single, although additional foci might have been located with more complete sectioning of the glands. In 63 goats, the tumors were multiple, with lesions present in both the right and left glands in 58 of these. Large tumors rarely were the only one in a gland and numerous smaller foci almost always were present.

Microscopic Appearance

In this series, many stages in the development of adrenal cortical adenomata were present, from the appearance of the first few neoplastic cells to the larger tumors. Because of this and the large number of cases, it becomes possible to recognize the small lesions which later mature into well-developed tumors and to trace many steps in the pathogenesis of the lesion.

In general, the microscopic appearance of the lesions varied with the size and, perhaps, the age of the lesion. The cells making up the formations varied from large polygonal to elongated spindle forms, with the polygonal type most frequently found in the smallest lesions. The smallest lesions usually were located near the capsule of the adrenal in the zona glomerulosa or just beneath it (fig. 4). Few were found in the deeper layers of the zona fasciculata or the zona reticularis, but occasional small lesions developed in the glomerulosa tissue found around the central veins.

The small nodules were made up of large irregular polygonal cells which bore some resemblance to the cells of the zona fasciculata (fig. 2, 3, 4). Great variation existed in the staining, the size, and the shape of the tumor cells. Their light pink to deep pink cytoplasm was foamy and variable in density. Their nuclei were large and oval with loosely scattered chromatin material and large distinct nucleoli (fig. 3). There was variation in the size of the nuclei with many doubly nucleated cells. Many nuclei contained several nucleoli. The interstitial tissue was scanty and no capsule

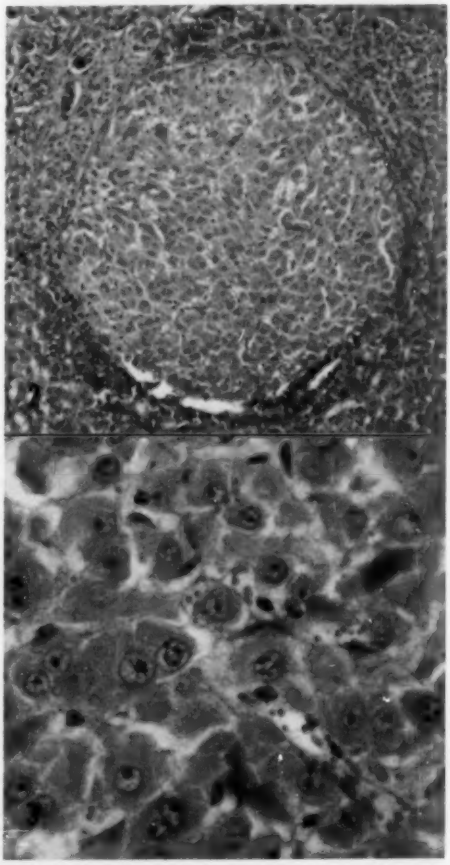

Fig. 2—Small cortical adenoma located in the outer adrenal cortex of a goat. Notice the size, shape, and irregular arrangement of the cells, as compared with the surrounding zona fasciculata. G1195, H & E stain; x 104.

Fig. 3—A higher magnification of an adenoma similar to that seen in figure 2. Cells are irregular and polygonal with foamy cytoplasm; notice the large distinct nucleoli and the inclusion body in a central nucleus. G1089, H & E stain; x 473.

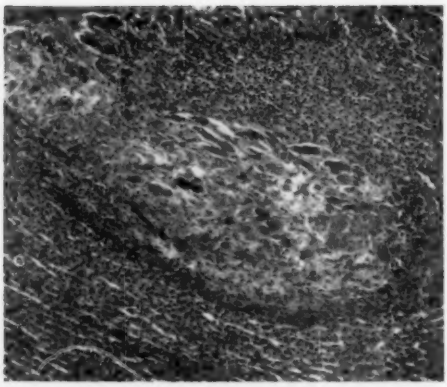

Fig. 4—A developing adenoma from a goat, with polymorphic and polychromatic cells. The adrenal capsule is at upper limits of picture and the zona fasciculata surrounds the lesion. G1086, H & E stain; x 40.

was present. The surrounding cortex usually was compressed, with cords of fasciculata cells outlining the lesion and setting it off as a distinct nodule (fig. 2, 4).

Larger lesions were located closer to the center of the gland and were associated with even greater compression of the surrounding cortex. These lesions seemed to extend farther into the center of the gland as they grew. Some of the largest tumors were surrounded by thick capsules of adult fibrous connective tissue. Perhaps this connective tissue originally was cortical interstitial tissue which gradually collected around the expanding lesion as the nearby cortical cells were destroyed by the resulting pressure.

Subsequent cellular growth of the lesion fell into two distinct patterns. In a few cases, there was a simple multiplication of the cell types just described, with the formation of a large adenoma consisting entirely of large foamy polygonal cells. These cells were well packed, arranged in an irregular pattern, and varied in size and staining characteristics. Doubly nucleated cells and mitotic figures were present in greater numbers than in normal cortices. Occasional nuclei contained large round inclusion bodies which stained a pale blue with hematoxylin and eosin (fig. 3). With the exception of the nuclear changes, these formations had a striking resemblance to normal lutein tissue with respect to size, shape, arrangement, and staining of the cells.

Larger lesions of this type contained signs of rapid uncontrolled growth, with cellular changes that might be indicative

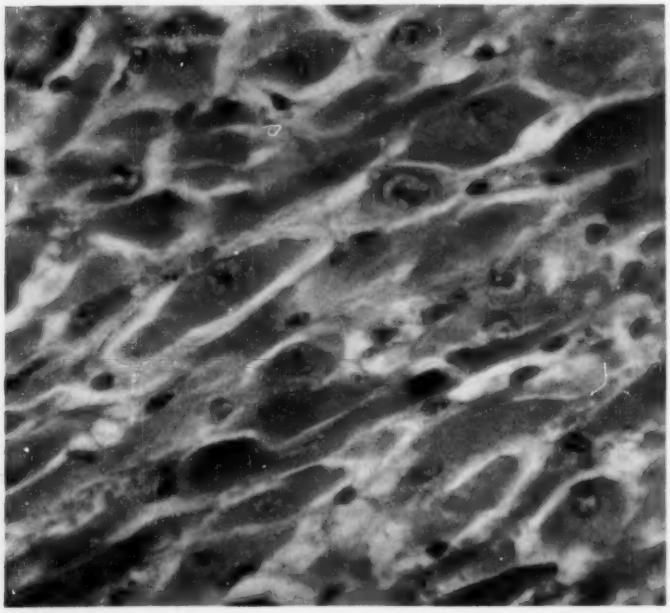

Fig. 5—Neoplastic cells which are slightly fusiform. They contain the foamy cytoplasm and oval nuclei typical of the polygonal cells shown in figure 3. G820, H & E stain; x 650.

of malignancy in adrenal tumors of other species. Although complete gross and microscopic necropsies were performed on all of the goats, there were no metastases of the adrenal capsule. Many cells of these nodules were large, irregular in outline, and filled with a dense deeply staining and often vacuolated cytoplasm (fig. 6, 7). The nuclei often were lobulated or multiple, with internal vacuoles and inclusion bodies. The lobulated nuclei were of a deep color and densely packed with chromatin material; other nuclei were large oval structures which ranged up to 25 or 30 μ in diameter. In these large nuclei, chromatin material was concentrated along the nuclear wall and loosely scattered internally. Numerous mitotic figures, many of which were abnormal, were seen in the nuclei of these adenomata.

The other course of development taken by the lesion was more commonly observed. In these instances, some of the polygonal cells elongated into narrow spindle forms (fig. 5). Cells of older or larger lesions were longer and narrower (fig. 9). The fusiform cells were usually arranged in whorls and were intermixed with clumps of polygonal cells (fig. 8). The polygonal types were similar to those described previously, and resembled cortical cells. The spindle types did not resemble cortical cells but, by examination of lesions in all stages, a course of development of fusiform cells was established. This development began with cells resembling cortical cells. The fusiform types had a scanty cytoplasm drawn out into thin, lightly stained strands with indistinct cell limits and walls (fig. 9). The nuclei were small, oval, or slightly elongated bodies with evenly distributed chromatin material. Mitotic figures were not prominent. Only rarely were all of the cells fusiform.

For convenience of study, the adenomata in this series have been grouped roughly into three categories, as determined by their cell type. This is done to indicate the relative incidence of the variations. Of the 113 specimens, 44 contained only the polygonal cells, and the majority of these were the smaller developing tumors; 63 had both polygonal and spindle cell forms intermixed with each other, including those lesions in which the polygonal cells were just beginning to lengthen. In those glands with tumors of mixed cell type, there often were smaller developing tumors consisting entirely of polygonal cells. Only six lesions were made up entirely of spindle cells. The fusiform cells were arranged in whorls and contained scanty cytoplasm and small dark nuclei.

The oil red O-stained sections did not illustrate any definite pattern of lipid distribution. Normal goat adrenals contained small amounts of lipid in the zona glomerulosa but were almost devoid of this material in the zona fasciculata. Some tumor nodules contained more lipid material than the nearby cortex and others contained less. Lesions in which spindle cell types

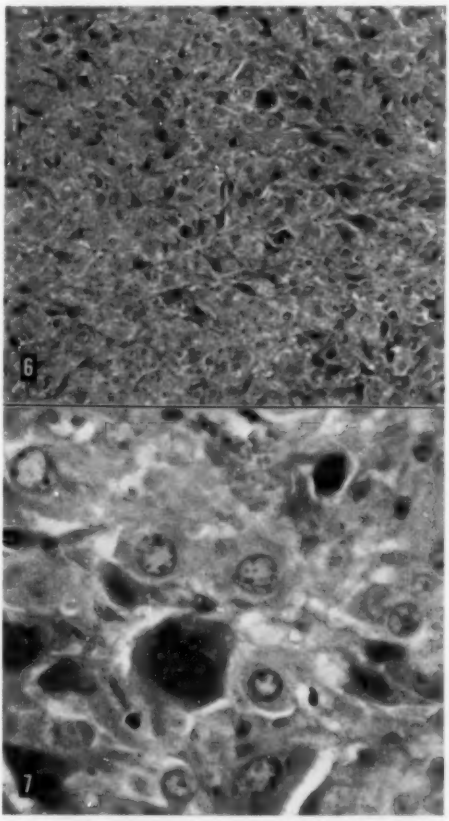

Fig. 6—An adenoma from a goat, made up of extremely variable cells. Notice the large hyperchromatic cells and nuclei. G1086, H & E stain; x 105.

Fig. 7—A higher magnification of figure 6, illustrating the extreme variation in cells and nuclei. G1086, H & E stain; x 472.

were prominent usually contained less lipid than the polygonal types, but this was not always true.

INCIDENCE

The distribution of these adrenal cortical adenomata according to sex is of interest (table 1). No adrenal cortical adenomata were found in the 23 adrenals from male Angora goats or in 221 glands from dairy breed males. Tumors were found in only two of 267 adrenals from female dairy goats and none were seen in six glands from female Angoras. Thus, there was a 0.73 per cent involvement of the adrenal glands obtained from the female goats. There was no clinically evident endocrine disturbance in either of the 2 female goats with the adrenal cortical tumors.

The incidence in castrated male animals was strikingly higher in both the Angora and dairy breeds. Of the 666 adrenals of castrated male Angoras, 157 (23.6%) contained cortical adenomata. These tumors were present in 102 of the 364 wethers examined. In the dairy breeds, 104 adrenals from 71 castrated males were examined and 12 (11.5%) of the glands were neoplastic, tumors being present in 9 of these goats. There was a 21.9 per cent involvement for all glands taken from castrated goats. Percentage figures for the animals involved would be misleading because both adrenals were not examined in all cases.

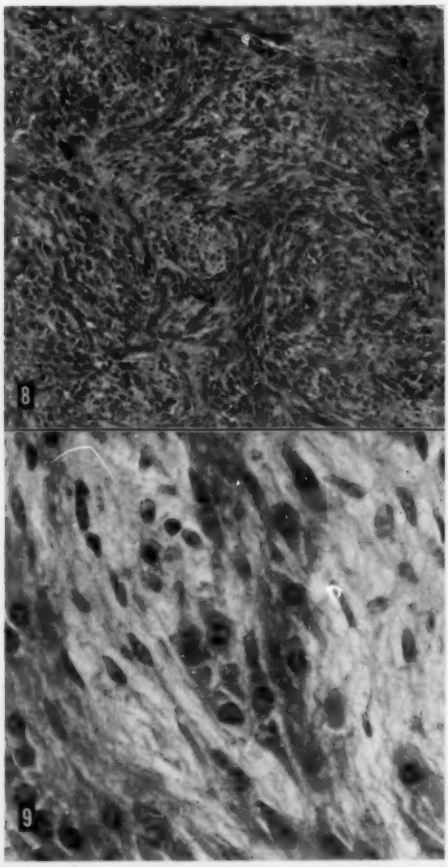

Fig. 8—An adenoma from a goat, consisting of spindle cells arranged in whorls and intermixed with polygonal cells. G836, H & E stain; x 114.

Fig. 9—A higher magnification of the fusiform cells seen in figure 8. G836, H & E stain, x 495.

DISCUSSION

Because of the high incidence of adrenal cortical tumors in cattle and sheep, it is not surprising to observe large numbers of these tumors in other ruminants. The lack of previous reports for other ruminants, such as the goat, probably is due to the fact that few of these animals are examined routinely. Except for small laboratory animals, there is little information about the relationship of castration and adrenal cortical tumors. In the domestic ruminants, it is rare to find castrated males that are allowed to live for many years after the loss of their testes. These animals are killed at a relatively young age. The Angora goat[16] provides an excellent example of a male ruminant which, in the course of routine husbandry methods, is castrated at an early age and allowed to live for a number of years. These wethers are used for the production of mohair until they no longer produce a quality product. They are then used as experimental animals in our laboratory; most are over 4.5 years old. Thus we have an experimental ruminant which was castrated early in life and then allowed to live for four to five years. We have no reliable information on the age of castration in the dairy breeds, but the lower incidence of adrenal cortical neoplasia might indicate that they have not lived as long following castration.

All of the tumors reported here were small when compared to those reported in cattle and sheep.[5,10] It is not known whether tumors of similar size develop in

goats, but it is possible that these small tumors might have grown to such size had the animals lived longer. Also, most reported tumors have been found at slaughterhouses as a result of routine inspection and only adrenals which were grossly abnormal would be examined microscopically. Perhaps many small neoplastic foci would be present in glands examined microscopically, even though they were grossly normal.

These findings in the goat support the findings on the production of adrenal cortical tumors in laboratory animals. Several nonruminant species of animals (the horse, dog, cat) are often castrated and allowed to live for a number of years. These animals might provide an additional source of information on the relationship of gonadectomy and the development of adrenal cortical tumors. However, these species seem to be less prone to develop adrenal neoplasms.

SUMMARY

The adrenal glands from 831 goats were examined for cortical adenomata. It was found that 169 of 770 adrenals from castrated males had these tumors, while 244 glands from male goats were free of them; only two of 273 glands from females were involved. These tumors are described and their incidence and probable pathogenesis are discussed.

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The Successful Control of Hog Cholera Virus Leukopenia with *Hemophilus Pertussis* Bacterin

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VACCINATION with the virus of hog cholera usually is followed by a severe leukopenia within one to nine days. This reduction in circulating leukocytes is believed by some to be an undesirable sequel to vaccination.

Dinwiddie,[2] in 1914, called attention to leukopenia as constituting the most obvious change in the blood picture of cholera-affected swine. Lewis and Shope,[4] in 1929, studied the cells of the blood in swine with hog cholera and concluded that a drop below 8,000 leukocytes per cubic millimeter was diagnostic. Cole[1] found that injection of anti-hog cholera serum alone did not reduce the leukocyte count, but that serum and cholera virus simultaneously did result in a reduction in the leukocyte count.

Lewis and Shope,[5] during investigations of "postvaccination breaks," inoculated 2 swine with a culture of the "bipolar organism" isolated from animals with pasteurellosis and found that a sustained leukocytosis followed. However, when they inoculated the bipolar organisms into hogs with cholera which had leukocyte counts of 3,000 to 4,000 per cubic millimeter, "instead of exhibiting an increase in leukocytes as might perhaps be expected," a more marked leukopenia resulted promptly.

Kernkamp,[3] in 1939, reported a comprehensive study of the blood picture in pigs with naturally occurring and induced hog cholera. He found an average preinfection leukocyte count of 14,200 in pigs with induced cholera and concluded that 8,000 leukocytes per cubic millimeter or less was abnormally low (leukopenia). The finding of 3 or more pigs with leukopenia in a herd was considered a valuable aid in making a diagnosis of hog cholera.

Inoculation with *Hemophilus pertussis* bacterin is known to produce an increase in the leukocyte count (leukocytosis) in the routine vaccination of children. This report summarizes a study of the cytological effect on the leukocytes in the young pig of *H. pertussis* bacterin injected alone and simultaneously with and at varying intervals after injection of hog cholera virus. Some observations of immunity to hog cholera are included.

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MATERIALS AND METHODS

The *H. pertussis* bacterin used was that commercially available or one obtained from the North Carolina State Public Health Department, and contained approximately 20 billion organisms per milliliter.

Pigs 8 to 12 weeks of age were obtained from the college herd and local farms, and would be classed as average farm pigs unless otherwise specified. Pigs were fed an 18 per cent protein "pig-growing" ration and were allowed to graze Ladino clover and grass pasture (trials 1, 3) or were housed in isolation units with air lock-type entrances (trials 2, 4, 5). Both spring and fall litters were used. The study was begun in 1953 and extended over a three-year period.

Blood samples were taken with leukocyte pipettes directly from a venipuncture of the ear veins. Differential leukocyte counts were made in all pigs in trials 1, 2, and 3, but were discontinued because values fluctuated without apparent reason and were considered unimportant. Pretreatment leukocyte counts for two to five days were obtained in each trial. Rectal temperatures were taken daily. Modified live hog cholera virus (porcine, lapine, and tissue culture origin) was given intramuscularly and *H. pertussis* bacterin subcutaneously.

TABLE 1—Leukocyte Counts of Pigs in Trial 1 Before and After Experimental Inoculations

Day of trial	Leukocytes (thousands/cmm.)	
	Pig 1	Pig 2
1	26.0	25.7
3	20.4	23.5
5	19.0	21.6
8	19.3	23.3
10*	29.2	24.9
11	26.3	26.8
12	25.5	25.8
13	19.5	20.5
14	19.9	20.0
18†	21.0	28.8
19	39.6	33.0
20	45.3	36.8
21	21.6	29.1
22	20.1	26.7
25**	.....	.....
26	42.6	34.6
27	25.2	38.1
28	17.6	31.2
29	25.6	34.2
31	21.8	20.9
32	22.6	26.2

* Given 1.0 ml. of *Hemophilus pertussis* bacterin; † given 1.5 ml. of *H. pertussis* bacterin; ** given 1.5 ml. of *H. pertussis* bacterin and 2.0 ml. of modified hog cholera virus.

TABLE 2—Leukocyte Counts and Survival Data for Pigs in Trial 2 Before and After Vaccination with Modified Hog Cholera Virus and Hemophilus Pertussis Bacterin

Day of trial	Leukocytes (thousands/cmm.)							
	Group A pigs (No.)				Group B pigs (No.)			
	1	2	3	4	5	6	7	8
1	.....	26.6	19.9	19.2	22.5	20.0	25.6	.....
3	.....	25.2	29.4	26.3	18.9	20.1	23.0	31.1
4	36.0	20.9	18.6	.....	25.3	.....	23.1	20.2
5	28.0	C	19.9	30.7	.....	19.1	36.3	30.3
6*	25.2	24.4	23.7	35.7	28.1	18.9	26.6	23.7
7	92.0	49.9	49.9	41.4	22.7	22.2	21.8	19.8
8	55.7	53.7	57.8	64.1	24.5	23.1	32.6	51.0
9	25.3	25.6	28.6	27.3	11.3	11.5	24.1	24.5
10	12.7	C	8.6	23.6	8.9	7.8	11.6	18.9
11	14.1	12.8	14.4	20.5	10.0	15.3	13.7	17.5
12	16.4	15.6	6.8	13.2	4.5	14.5	8.6	12.2
13	16.3	9.6	16.8	18.7	20.0	9.1	16.0	10.0
14	17.9	11.4	19.4	17.2†	23.4	5.6	23.3	12.8
15	11.7	5.5†	21.9	35.3	24.4	4.9	35.0	13.0
16	8.3	6.6†	21.4	32.0	23.9	9.7	28.4	6.1
17	8.5	7.2	28.0	34.4	25.9	9.2†	26.7	5.8†
	Died	Died	Died	Lived	Lived	Died	Lived	Died

* Pigs in group A were given 2 ml. of *H. pertussis* bacterin and 2 ml. of modified virus; those in group B were given 2 ml. of modified virus. † Pigs had diarrhea.
C = clotted.

EXPERIMENTAL PROCEDURE AND RESULTS

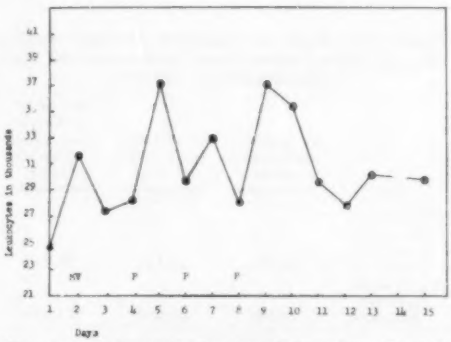
Trial 1.—Two weanling pigs were injected with *H. pertussis* to get an indication of the type and duration of the leukocyte response. There was a mild febrile response of 1 or 2 degrees. While the initial dose of 1.0 ml. of *H. pertussis* did not result in a significant increase in leukocytes. there did seem to be a sensitizing effect. Subsequent doses produced up to a 100 per cent increase in the leukocyte count, as compared with the prevaccination average. The peak effect was reached in one or two days, with a rapid recession to within normal ranges on the third day. The leukocyte counts, after two inoculations with *H. pertussis* bacterin followed by a simultaneous inoculation of *H. pertussis* and modified live virus without serum, are shown (table 1). Both pigs survived.

Trial 2.—Eight weanling pigs were used to evaluate further the effect on the leukocyte count of *H. pertussis* given alone and simultaneously with modified hog cholera virus. These pigs were lightly infested with lice and moderately infected with ascarides. Leukocyte counts were made for five days before inoculation. Four of the 8 pigs (group A) were inoculated with 2.0 ml. of modified virus; the other 4 (group B) were inoculated with 2.0 ml. of modified virus and 2.0 ml. of *H. pertussis*. The average leukocyte counts and survival data are summarized (table 2).

Trial 3.—This trial was designed to investigate further the objective stated for trial 2. Eight weanling pigs were purchased from a local farm; each was treated for endoparasites and ectoparasites, using oil of chenopodium and dichloro-diphenyl-trichloro-ethane (DDT*). Leukocyte counts were made for two days, and each pig then was vaccinated with 2.0 ml. of modified hog cholera virus. On postvaccination days 2, 4, and 6, each pig was inoculated subcutaneously with 2.0 ml. of *H. pertussis* bacterin. Leukocyte counts were taken daily for 13 days and the average count is shown (graph 1). All pigs survived.

Two of these pigs were selected at random 124 days after vaccination and given a chal-

* DDT is produced by Prentiss Drug and Chemical Co., Inc., New York, N. Y.



MV = 2.0 cc. of modified live hog cholera virus; P = 2.0 cc. of *H. pertussis* bacterin subcutaneously.

Graph 1—Leukocyte count averages on 8 pigs in trial 3 and dosage schedules of modified live hog cholera virus and Hemophilus pertussis bacterin.

TABLE 3—Leukocyte Counts of Pigs in Trial 4 Before and After Experimental Inoculations, and Results of Immunity Test

Day of trial	Leukocyte counts (thousands/cmm.)				
	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5
1	15.1	20.0	20.4	19.7	18.6
2	40.3	33.5	26.0	31.3	38.1
3	18.4	23.2	26.6	17.5	28.8
7*	15.1	20.3	19.6	20.1	19.6
Average	22.2	24.2	23.1	22.1	26.2
8	18.3	23.3	24.7	19.8	21.2
9	19.0	20.1†	24.1†	18.3†	15.7
10	19.9	15.4	22.5	19.6	16.4
13	23.5	8.5	23.2	20.5	33.0
14	23.1	7.8	22.7	22.1	24.4
15	22.9	5.6	22.5	24.0	20.1
16	15.0	7.6**	42.3	16.4	21.7
17	16.1	11.8	24.8	19.2	22.8
18	17.3	16.1	24.7	21.7	21.1
20	25.2	15.1	27.0	26.9	40.9
22	24.0	17.5	24.3	25.8	31.0
24	23.3	19.1	22.2	25.2	27.3
Challenge results‡	Died	Lived	Died	Lived	Died

* All pigs given 2 cc. of modified hog cholera virus; † given 2 cc. of *H. pertussis*; ** given 1 cc. of *H. pertussis*; ‡ two months after virus vaccination, each pig was challenged with 5 ml. of virulent virus.

lenge dose of 5.0 ml. of a commercially available virulent hog cholera virus. One died seven days and the other ten days after challenge. Both had an increase in rectal temperature and a leukopenia from the second day after challenge until death. The clinical, laboratory, and necropsy findings were classical for hog cholera.

Trial 4.—Five weanling pigs were used to obtain more information on the influence of dosage of *H. pertussis* on the leukocyte count and on the immunity produced by modified virus. They were vaccinated on day 7 with 2.0 ml. of modified virus. On day 2 after vaccination, pigs 2, 3, and 4 were injected with 1.25 ml. of *H. pertussis*. Pig 2 was inoculated again with 1.0 ml. of *H. pertussis* on day 6 after vaccination. The leukocyte counts are shown (table 3).

TABLE 4—Trial 4—Leukocyte Averages Before, On, and After Date of Virus Vaccination and Their Relationship to Survival

Pigs	Av. leukocyte count before virus inoculation	Av. leukocyte count on day of virus inoculation	Av. of 12 leukocyte counts over 17 days after virus inoculation
3 pigs which died following challenge	23,904	18,100	23,703
2 pigs which survived challenge	23,237	20,200	17,818

The immunity of the 5 pigs was challenged with 5 ml. of commerical virulent hog cholera virus two months later. Of the 3 pigs given modified virus followed by *H. pertussis*, 2 survived the challenge. The other 2 pigs, given modified virus only, died after challenge. The clinical signs and necropsy findings of the three that died were classical for hog cholera.

The relationship of leukocyte averages to survival is shown (table 4). The average leukocyte counts of the pigs that failed to survive challenge showed no appreciable changes after vaccination.

The 2 pigs that survived challenge showed a 23 per cent decrease in their average leukocyte counts after vaccination.

Trial 5.—Eighteen pigs were used to test further the influence on the leukocyte count of the time and quantity of *H. pertussis* injected and to compare the leukocyte counts after injection with virulent virus. Two pigs (group A) were placed in an isolation unit and injected with 1.0 ml. of virulent hog cholera virus. The other 16 were given 2.0 ml. of modified virus and were placed in groups B (8 pigs) and C (8 pigs). Each pig in group B subsequently was injected with *H. pertussis* as follows: 0.8 ml. on postvaccination day 2, 1.0 ml. on day 4, 1.0 ml. on day 6, and 1.6 ml. on day 8. Both pigs in group A developed hog cholera; 1 died on postvaccination day 15 and the other was killed for necropsy when moribund on day 15. All pigs of group B survived the experimental inoculations. Two pigs of group C died 18 and 20 days, respectively, after being given modified virus. The average leukocyte counts of groups in this trial are shown (graph 2).

Discussion

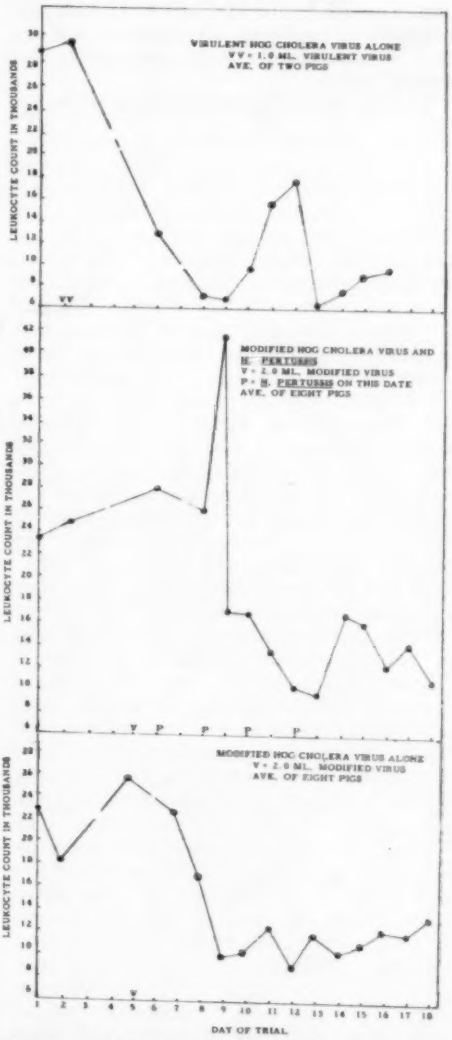
The data from trials 1 and 2 show that *H. pertussis* bacterin is a potent agent for increasing the leukocyte count in the young weanling pig. However, the effects of a single dose were highly transitory. Since deworming would not affect migrating larvae, parasitism could be related to or could be the primary cause of death losses in trial 2. The parasite load and the stress of vaccinations probably were additive.

Trial 3 demonstrated the ability of *H. pertussis* bacterin to prevent the development of leukopenia when injected subcutaneously two, four, and six days after vaccination with modified live virus. That neither of 2 of the 8 pigs selected at random withstood challenge with virulent hog cholera virus indicated the failure of the animals of the group to properly develop immunity. Since leukopenia did not develop after vaccination with the modified virus, the data from this trial also indicate that the hematological condition of leukopenia is favorable for the development of immunity with this virus.

It is of interest that a single dose of an *H. pertussis* bacterin was sufficient to offset a marked leukopenia in 2 of 3 pigs in trial 4. In some instances, there was a considerable difference in the response to *H. pertussis* inoculation by individual pigs in the same group. The leukocyte count increased more in some pigs than in others. The data in table 4 indicate the possibility that a strong immunity develops in weanling pigs vaccinated with modified virus only when there is a significant reduction of leukocytes for a few weeks after inoculation.

In trial 5, the degree of leukopenia varied among individual pigs on a given day. Cole [1] also found that leukopenia was not marked in all individual pigs acutely affected with induced hog cholera. The mobilizing influence of *H. pertussis* on the leukocyte counts is demonstrated in pigs of group B. A comparison of the counts in pigs of groups C and A shows that the extent of reduction of leukocytes is similar. However, the severe depressant action of virulent virus seems to have been more prolonged.

Pigs given virulent virus alone usually showed a severe leukopenia followed by a marked rise in the leukocyte count at about the time the diarrhea appeared. It is possible that pigs which do not show a marked leukopenia (under 8,000/cmm.) when hog cholera virus alone is injected, may harbor a coexisting bacterial infection that tends to elevate the leukocyte count and thus offsets partially the full leukopenic effect of the virus. Such a bacterial infection could exert an inhibitory effect on the developing immunity in some pigs.


VIRULENT HOG CHOLERA VIRUS ALONE
V V = 1.0 ML. VIRULENT VIRUS
AVE. OF TWO PIGS
MODIFIED HOG CHOLERA VIRUS AND
H. PERTUSSIS
V = 2.0 ML. MODIFIED VIRUS
P = H. PERTUSSIS ON THIS DATE
AVE. OF EIGHT PIGS
MODIFIED HOG CHOLERA VIRUS ALONE
V = 2.0 ML. MODIFIED VIRUS
AVE. OF EIGHT PIGS
LEUKOCYTE COUNT IN THOUSANDS
DAY OF TRIAL

Graph 2—Effects on the leukocytes in weanling pigs in trial 5 of virulent hog cholera virus, modified hog cholera virus, and simultaneously given modified virus and Hemophilus pertussis.

CONCLUSIONS

1) *Hemophilus pertussis* bacterin produced a marked leukocytosis when injected subcutaneously in the normal weanling pig.

2) The pattern of leukopenia that usually follows vaccination with modified hog cholera virus can be altered considerably by appropriately timed injections of *H. pertussis* bacterin.

3) *Hemophilus pertussis* injected by the subcutaneous route and given at two-day intervals after virus vaccination can be used to maintain the leukocytes within the normal range.

4) The hematological condition of leukopenia that usually follows vaccination with modified hog cholera virus appears to be necessary for the development of a strong immunity.

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Inclusion Body Rhinitis of Swine in Maryland

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INCLUSION BODY RHINITIS of swine (IBR) was first described in Great Britain, in 1955, by Done,[2] who reported that typical lesions could be demonstrated in the early stages of naturally occurring and experimentally induced atrophic rhinitis, and that atrophic rhinitis could be produced in susceptible young pigs by the intranasal instillation of material from animals with inclusion body rhinitis.

The letters, "IBR," originally used by Done to denote inclusion body rhinitis, subsequently have been used by McKercher *et al.*[5] to denote infectious bovine rhinotracheitis. The double usage may lead to some confusion. In this report, "IBR" is used in its original connotation, and the letters "AR" are used to denote atrophic rhinitis.

MATERIALS AND METHODS

To determine whether IBR occurs in the United States, material was examined from pigs in the experimental breeding herd at the Agricultural Research Center, Beltsville, Md. Atrophic rhinitis is known to have been enzootic in this herd for at least five years. The material examined earlier was taken from pigs killed or dying of a variety of incidental causes. Later, 3 sows were selected by rhinoscopic examination as having AR, and pigs from their litters were killed at intervals.

The snouts of the pigs were cut transversely at about the level of the first premolar, and the macroscopic appearance of the nasal cavity was observed. The ventral turbinates then were removed and fixed, some in 10 per cent formalin solution, and others in a modification of Bouin's fluid.* Formalin-fixed material was decalcified by a proprietary decalcifying mixture.† Blocks of tissue embedded in paraffin were cut at a thickness of about 6 μ. Sections from all specimens were stained by Harris' hematoxylin and eosin and, in some cases, by Giemsa's stain.

HISTOPATHOLOGY

The pathognomonic lesions of IBR are found in the glands of the lamina propria and in their ducts. The cells and their nuclei become markedly enlarged. A well-defined nuclear membrane, within which lies a large, basophilic, finely granular body, is visible at low magnifications (fig. 1). Some of these bodies are round and completely separated from the nuclear membrane; others are irregularly indented and connected to the nuclear membrane by fine strands. It occasionally is possible to demonstrate small, more neutrophilic spheroids lying close to the basophilic inclusion. In early cases, the cytoplasm of the affected cell appears swollen and ballooned; later, the line of division between the cytoplasm of adjacent cells is lost, and the inclusion-bearing nuclei are seen scattered among irregular shreds of eosinophilic necrotic cytoplasm.

The changes in the nuclei of the affected cells are accompanied by round cell infiltration of the lamina propria, made up predominantly of lymphocytes accompanied by a much smaller number of plasma cells and macrophages; occasionally, clumps of eosinophils also are seen. The infiltration may be slight at the time of the first appearance of the inclusion bodies, but rapidly becomes more intense and obliterates many of the gland masses. The lumina of some of the glands and ducts sometimes contain irregular plugs of necrobiescent polymorphonuclear leukocytes, but the infiltration of the lamina propria usually is nonpurulent. At the same time, the epithelium often loses its cilia and takes on a stratified squamous appearance.

The processes in the lamina propria commonly are accompanied by some thinning and distortion of the bony core. In some cases, a few inclusions may still be present when areas of complete fibrous

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* Formalin, 100 cc.; glacial acetic acid, 50 cc.; picric acid, 12.2 Gm.; water, 900 cc.

† Decal, produced by the Omega Chemical Co., Inc., Long Island, N.Y.

replacement of the bony core (typical of AR) have appeared. Downgrowths of the epithelium which appear in the later stages probably differentiate to replace the glands destroyed by the earlier stages of the infection.

OBSERVATIONS

All the young pigs examined could have been exposed to infection from a number of sources, as they mingled with three or more litters in addition to having contact with their dams.

Fig. 1—Transverse section of the ventral turbinate of pig 1712, showing ballooned cells in the glands and ducts with enlarged nuclei and basophilic inclusions visible at low magnification. H & E stain; x 85.

Fig. 2—Transverse section of the ventral turbinate of pig 1627, showing fibrous replacement of bony core, typical of atrophic rhinitis. Small numbers of typical inclusions were still present, but are not visible at this magnification. H & E stain; x 35.

—AFIP Neg. 57-16783 (fig. 1); Neg. 57-16788 (fig. 2)

In the first stage of the investigation, 17 pigs, 13 to 77 days old, were examined as they died from a variety of causes. Macroscopically, the turbinates appeared normal in 12 pigs, in 4 there was evidence of shrinkage, and 1 showed doubtful signs. Microscopically, 11 appeared normal, 1 showed doubtful signs, 1 showed lesions of IBR alone, 2 of IBR and AR, and 2 of AR alone (table 1).

In the second stage, 19 pigs from three selected litters were examined.

Litter 1.—The pigs killed at 19, 26, 33, and 42 days of age appeared normal (table

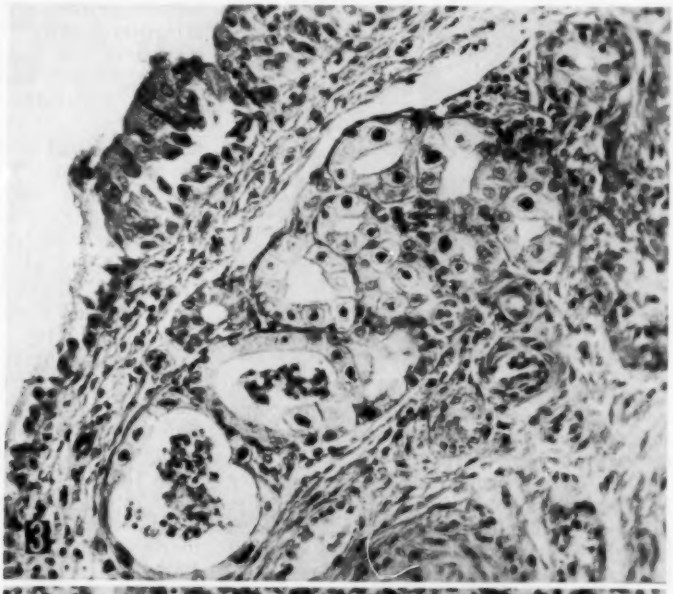

Fig. 3—Section from pig 1712, showing an area from figure 1. Notice the masses of necrobiescent leukocytes in the duct lumina. H & E stain; x 265.

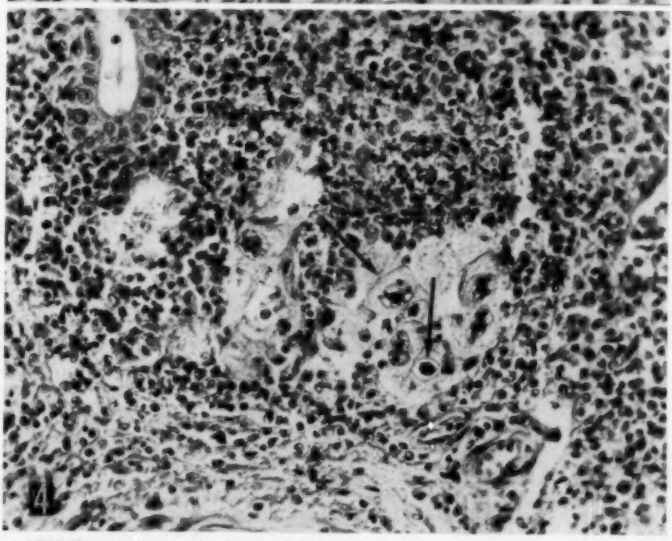

Fig. 4—Section of the ventral turbinate of pig 1620. Both round and stellate forms of the inclusion bodies are visible (arrows). The cytoplasm of affected cells is beginning to coalesce. There is heavy infiltration of round cells, some of which show pyknosis and karyorrhexis. H & E stain; x 265.

—AFIP Neg. 57-16784 (fig. 3); Neg. 57-16786 (fig. 4)

TABLE 1—Evidence of Inclusion Body Rhinitis and Atrophic Rhinitis in 17 Unselected Pigs

Pig (No.)	Age* (days)	Macroscopic evidence of turbinate shrinkage	Microscopic diagnosis	Remarks
206	13	—	—	....
141	16	—	—	....
181	16	—	—	....
9884	23	—	—	....
9972	24	—	—	....
572	24	—	IBR	....
9956	25	—	—	Small abscess in lamina propria
1D	35	+++	AR	....
9283	40	—	—	....
9895	41	—	—	....
3D	42	++++	(IBR) (AR)	Severe loss of bone
4D	42	++	(IBR) (AR)	....
637	48	—	—	....
9191	58	±	±	Some round cell and eosinophil infiltration
9461	58	++	AR	....
9885	58	—	—	....
9287	77	—	—	....

* When killed for necropsy.

2) except for the 26-day-old pig, in which there was a suggestion of macroscopic shrinkage of the turbinate but no obvious microscopic abnormality.

There was no macroscopic abnormality in the 48-day-old pig. Microscopically, patchy round cell infiltration of the lamina propria was marked, with areas of glandular destruction and some squamous metaplasia of the surface epithelium. Many gland and duct cells contained basophilic intranuclear bodies, but without marked enlargement of the nuclei. The bony core was narrowed in places, but areas of total fibrous replacement were not seen.

The 55-day-old pig had a degree of macroscopic shrinkage of the turbinates. Microscopically, patchy round cell infiltration of the lamina propria, some destruction of glands, squamous metaplasia of the epithelium, and a few typical inclusions were visible. The bony core was narrowed. The 62-day-old pig had moderate macroscopic shrinkage of the turbinates. Microscopically, the lamina propria was infiltrated by round cells. Squamous metaplasia of the epithelium and areas of complete fibrous replacement of the bony core were evident.

The 71-day-old pig had no obvious macroscopic abnormality of the turbinates. Microscopically, the connective tissue stroma of the lamina propria was somewhat thickened and contained a moderate patchy infiltration of round cells and a small number of eosinophils. Squamous metaplasia had occurred in some parts of the epithelium. The bony core was narrow in places, but there were no areas of complete fibrous replacement.

Litter 2.—No abnormality was detected in pigs killed at 16 and 23 days of age (table 3). Slight shrinkage of the turbinates in the 30-day-old pig was evident macroscopically. Microscopically, the round cell population of the lamina propria was slightly increased, and much of the bone appeared cellular and immature. Some nuclei of gland cells contained basophilic bodies, but were not enlarged.

In the 37-day-old pig, shrinkage of the turbinates was marked. Microscopically, the lamina propria was infiltrated by numerous round cells and some eosinophils. Parts of the epithelium had undergone squamous metaplasia, and fairly numerous typical inclusion bodies were accompanied by cytoplasmic ballooning of gland and duct cells. The bone was extremely narrowed and distorted, but areas of total fibrous replacement were not visible.

In the 44-day-old pig, shrinkage of the turbinates was moderate. Microscopically, moderate round cell infiltration, a reduced number of glands, and increased density of the connective tissue were observed in the lamina propria. Large areas of bone had

TABLE 2—Observations in Litter 1, from a Sow Showing Evidence of Atrophic Rhinitis

Pig (No.)	Age* (days)	Macroscopic evidence of turbinate shrinkage	Microscopic diagnosis
1250	19	—	—
1254	26	+	—
1251	33	—	—
1257	42	—	—
1253	48	—	±
1256	55	+	(IBR ± AR)
1252	62	++	AR
1255	71	—	+

* When killed for necropsy.

TABLE 3—Observations in Litter 2, from a Sow Showing Evidence of Atrophic Rhinitis

Pig (No.)	Age* (days)	Macroscopic evidence of turbinate shrinkage	Microscopic diagnosis
1622	16	—	—
1627	23	—	—
1623	30	+	±
1620	37	+++	(IBR ± AR)
1624	44	++	AR
1628	52	++	(IBR) (AR)
1625	57	+++	AR

* When killed for necropsy.

undergone total fibrous replacement, and one of these contained a large group of osteoclasts.

The 52-day-old pig showed moderate macroscopic abnormality. Microscopically, there was moderate round cell and some eosinophilic infiltration, reduction in the number of glands, increased density of the connective tissue stroma, and a small number of typical inclusions. The bone had areas of fibrous replacement.

The 57-day-old pig had marked macroscopic shrinkage of the turbinates. Microscopically, the lamina propria was diffusely infiltrated with round cells, accompanied by considerable numbers of eosinophils. The number of glands was reduced and the epithelium showed large areas of squamous metaplasia. The bony core was narrowed and distorted, with large areas of complete fibrous replacement.

Litter 3.—Pigs killed at 25, 32, and 39 days of age showed no macroscopic abnormalities (table 4).

In the 39-day-old pig, however, there was patchy round cell infiltration of the lamina propria, and many gland and duct cells showed cytoplasmic ballooning and typical intranuclear inclusions.

In the 48-day-old pig, macroscopic shrinkage of the turbinates was questionable. Microscopically, a moderate patchy round cell infiltration was present in the lamina propria, and a number of gland cells contained typical intranuclear inclusions. Evidence of abnormality in the bone was equivocal.

The 52-day-old pig had questionable macroscopic shrinkage of the turbinates. Microscopically, the lamina propria was infiltrated with many round cells and a few eosinophils. The glands had undergone considerable destruction, and a moderate number of typical inclusions were visible. The bony core was much narrowed, but complete fibrosis was not demonstrable.

DISCUSSION

The lesions of IBR suggest that the infection may be caused by a virus belonging to the same group as those causing cytomegalia in man and other animals.[1,3-6] It generally is recognized[2,7] that lesions macroscopically resembling those of AR might be reproduced by the intranasal instillation of a number of agents capable of causing chronic inflammation in rapidly growing pigs. In this respect, AR should be thought of as a lesion, rather than as a specific disease. It seems possible that the agent of IBR may be the cause of at least some field cases of AR, and may be identical with the filter-passing agent described by Switzer.[7]

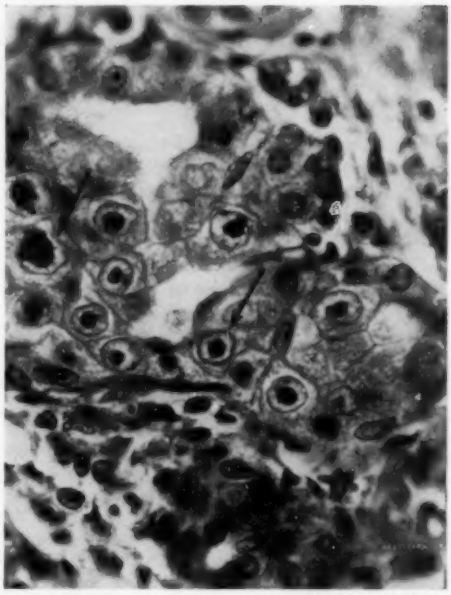

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Fig. 5—Section from pig 1620. Numerous gland cells show cytomegaly. Both round and irregular forms of inclusion bodies are visible (arrows). A few relatively normal cells remain. H & E stain; x 540.

In the limited number of pigs examined, the age incidence presented an irregular pattern, but it appears that the lesions of IBR usually are seen earlier than the changes taken as typical of AR. Assuming that the period of persistence of the inclusion bodies is relatively short, these two

TABLE 4—Observations in Litter 3, from a Sow Showing Evidence of Atrophic Rhinitis

Pig (No.)	Age* (days)	Macroscopic evidence of turbinate shrinkage	Microscopic diagnosis
1711S	25	—	—
1714	32	—	—
1712	39	—	IBR
1721	48	—	IBR
1719	52	±	(IBR ± AR)

* When killed for necropsy.

factors may explain why the condition has not been recorded previously in this country.

SUMMARY

Lesions histologically indistinguishable from those of inclusion body rhinitis (IBR) were found in a herd of swine in Maryland. The herd was known to be affected with atrophic rhinitis (AR).

Of 37 pigs, 13 to 77 days old, 9 (24–55 days old) showed lesions of IBR and 8 (35–62 days old) showed lesions of AR.

It is suggested that the causative agent of IBR is a member of the group of agents causing cytomegalia in man and a number of other animals, and may be a factor in the production of AR.

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Comparative Histopathology of Experimental Swine Influenza and Virus Pneumonia of Pigs in Disease-Free, Antibody-Devoid Pigs

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EARLY in his work with swine influenza, Shope [4] recognized the occurrence of more than one clinical entity associated with respiratory diseases of swine. A transmissible filterable agent from animals with typical cases of swine influenza was obtained easily in 1928, whereas much difficulty was encountered in obtaining infective material in 1929. Although cases were seen which simulated true epizootic swine influenza in some respects, they differed markedly in others. Greater chronicity, absence of prostration, and failure of more than a small portion of the herd to become afflicted were the distinguishing characteristics. It is likely that this more chronic respiratory disease of swine was virus pneumonia of pigs (VPP). Reviews of the literature on this disease have been made previously.[1, 2, 5]

The histopathology of swine influenza [4] and of VPP [3] have been described independently. A comparative study of these diseases in pigs, however, has not been reported. Since they have similar gross pathology, as well as superficially similar histopathology, this study was undertaken to evaluate possible histopathological differences.

MATERIALS AND METHODS

Experimental Pigs.—All pigs used in these studies were obtained two to four days prematurely by hysterectomy.[7] These pigs were handled so as to preclude exposure to respiratory infection and were housed throughout the experiments in modified Horsfall-Bauer isolation units.[6] They were considered disease-free and antibody-devoid. They were not given colostrum and were fed from birth on pasteurized, homogenized cow's milk fortified with eggs and minerals. The pigs were not germ-free, since they soon developed a bacterial intestinal flora derived from the pasteurized milk. Protozoa were not present. The pigs were approximately 1 week old when inoculated.

All 44 pigs were inoculated intranasally. Of these, 4 were given 5 ml. of nutrient broth and killed on the second (1 pig) and twenty-first days (normal controls), 4 were given the Shope strain 15 (S-15) virus which had been heated to 56 C. for four hours and were killed in pairs at 24 and 48 hours (egg inoculum controls), and 2 others were inoculated with the S-15 virus derived from pig lungs after two lung passages (virus source controls) and were killed four and 11 days after inoculation. Of the remaining 34 pigs, 17 were given the S-15 virus and killed singly or in pairs, one, two, three, four, five, seven, nine, 11, and 21 days after exposure, and 17 were given the VPP virus and killed singly or in pairs, three, seven, nine, 11, 13, 15, 17, and 21 days after inoculation.

Viruses.—The swine influenza virus used was Shope's strain 15 (S-15). The strain used in this laboratory had been passed 232 times in mice and 22 times in embryonating hen's eggs. Each milliliter contained approximately 10^7 $i.d._{50}$ for week-old, antibody-devoid pigs. This material was diluted 10^{-3}, and 5 ml. of the diluted virus suspension was given intranasally to lightly anesthetized (ether) pigs. Virus originating from swine lung as a virus source control was obtained by two successive passages of the S-15 egg virus in pigs.

The VPP virus used was strain NB12, isolated in this laboratory from the lung of a pig with respiratory disease and passed serially 13 times by intranasal instillation in disease-free pigs.

Virus material for inoculating experimental pigs was obtained by triturating swine lungs in a mortar with ground glass and was diluted with nutrient broth. The suspension was centrifuged at 3,000 r.p.m. for 30 minutes in an International No. 1 refrigerated centrifuge at 4 C. and treated with 1,000 units of penicillin and 1,000 μg. of streptomycin per milliliter for one hour at 4 C. The experimental pigs were inoculated with 5 ml. of a 10^{-3} dilution of virus material, given intranasally under light ether anesthesia.

Tissues.—Pigs were anesthetized with chloroform (at specified intervals after inoculation), and exsanguinated. Portions of lung, heart, liver,

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kidney, and spleen were placed in 10 per cent neutral formalin or Zenker's fixatives. Prepared sections were stained with hematoxylin and eosin.

RESULTS

The gross pathological changes in both diseases are similar. Generally, the apical and cardiac lobes of the lung were the only parts affected. Occasionally, the consolidated brownish red (plum-colored) areas may be scattered throughout all the lobes. This occurred more commonly in pigs with influenza that in those with VPP. Pigs with cases of longer standing may show slight depression in the consolidated areas, with the adjacent healthy tissue showing emphysema.

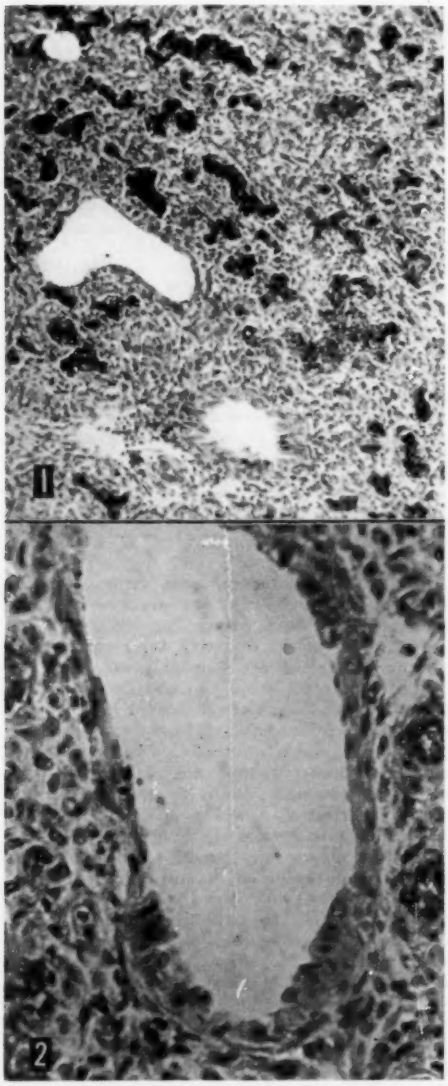

Fig. 1 and 2—Sections of lungs from pigs with swine influenza, on the sixth day after infection, showing (fig. 1) alveoli filled with cellular debris, and (fig. 2) bronchiolar epithelium with degenerative changes. Fig. 1, x 125; fig. 2, x 450.

In the experimental diseases, the bronchial lymph nodes responded differently. The S-15 virus stimulated an enlargement of the lymph nodes without alteration of color. In the case of VPP virus, there was no gross pathological change in the nodes.

Histopathology of Swine Influenza.—Tissue responses to the introduction of S-15 virus were rapid. Within 24 hours, there were scattered areas of edema and hyperemia throughout the lung. By the third day, the alveoli and bronchi contained many polymorphonuclear cells and many areas were filled with cellular debris (fig. 1). Degenerative and regenerative changes were apparent by the fourth and fifth days. The degenerating bronchial epithelium stained deeply eosinophilic, with evidence of damage to the nuclei (fig. 2). Accompanying regeneration showed hypertrophic epithelial cells with bluish cytoplasm. There was an above normal incidence of mitotic figures, and a few areas showed a slight lymphocytic perivascular and peribronchiolar reaction (fig. 3). Up to the sixth day, the mononuclear perivascular reaction was more pronounced than the peribronchiolar reaction (fig. 4). However, the number of lymphocytes surrounding the bronchi increased daily, so that peribronchiolar cuffing became the predominant lesion. Consolidated areas showed atelectasis. In these areas, some bronchi still contained cellular debris and the epithelial cells had degenerated or were necrotic.

Maximal cellular reaction occurred within 11 days. Heavy mononuclear cell infiltrations had occurred around the small bronchi by that time. Alveoli adjacent to these small bronchi contained alveolar lining cells, a few large mononuclear cells, and polymorphonuclear leukocytes. Interalveolar connective tissue was increased in amount and contained mononuclear cells. Giant cells were seen occasionally in some of these cases. Hyperplasia of the lym-

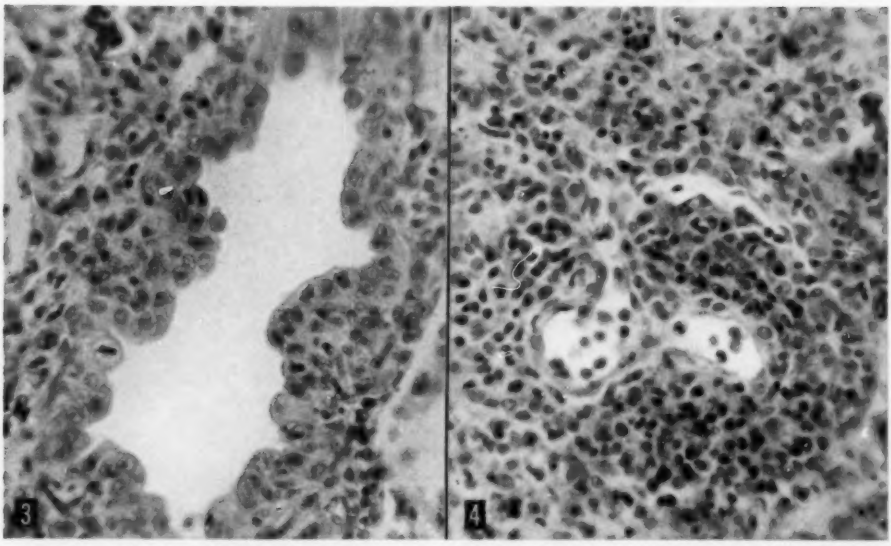

Fig. 3 and 4—Lung sections from pigs with swine influenza, on the fifth day after infection, showing (fig. 3) regenerating bronchial epithelium, and (fig. 4) perivascular round cell infiltration. x 400.

phatic tissue of the lymph nodes also was observed.

There was no essential difference in the histopathological changes in pigs with swine influenza when S-15 virus in allantoic fluid was used as inoculum, as compared with changes when S-15 virus originated from lung tissues.

Histopathology of Virus Pneumonia of Pigs.—Pigs inoculated with VPP virus and killed three, seven, and nine days later did not show any abnormal cellular reactions. At 11 days postinoculation, 1 pig showed marked hyperplasia of a lymph nodule between two bronchi, and lymphocytic cell infiltration within the propria mucosa of some larger bronchi (fig. 5). No reaction was observed at this stage in the alveolar parenchyma, although granulated plasma was found in some of the alveoli.

Pigs killed 13 or more days after exposure to VPP virus showed a consistent histopathological picture. The characteristic changes were severe perivascular and peribronchial round cell infiltration (fig. 6, 7). About half the pigs showed evidence of perivascular cuffing more severe than that surrounding the bronchi. The characteristic changes in these organs are described separately.

The endothelium of some of the vessels in the lung was hypertrophic and the lumina contained masses of small and large mononuclear cells. The perivascular cell reactions often were so severe that it was difficult to distinguish the vessels. The lumina of such vessels were almost closed by compression of surrounding round cells. Perivascular reactions did not occur in tissues other than the lungs.

In all pigs, there was a distinct peribronchial lymphocytic cell infiltration. The hyperplasia of the lymphatic tissue within the propria mucosa of the bronchi was so marked that the lumen was almost obstructed (fig. 8). Infiltrating cells separated the individual elements of the bronchial epithelium, causing the epithelial cells to disappear or to be seen only with difficulty. The muscularis mucosa was compressed and atrophic. There was no evidence of necrosis or regeneration. There was some increase in intra-alveolar connective tissue, which also contained lymphohistiocytic elements.

Discussion

In the consideration of respiratory diseases of swine, it is desirable to make a specific diagnosis. It is particularly important to distinguish between swine in-

fluenza and VPP, as the recommendations would be different. Influenza is characterized by recovery of the patient, involving an immune response and exclusion of the infectious agent. The animals may be retained with little concern for perpetuating the disease. However, the causative agent for VPP does not elicit an immune response and may persist indefinitely. Animals held over may serve as the source of infection of succeeding generations. Control of VPP necessitates some means of obtaining entirely new VPP-free stock.

The observations made in these studies would indicate that these two diseases can be distinguished histopathologically in an early period in experimental animals. Although both diseases stimulate small and large round cell infiltration with perivascular and peribronchiolar reactions, the reaction is more severe in VPP. Influenza causes a bronchopneumonia early, with severe polymorphonuclear leukocyte infiltration and necrosis of the bronchiolar epithelium, followed by regeneration and increase of round cells. Virus pneumonia of pigs does not cause necrosis, but the bronchiolar epithelium is partly detached, due to the heavy round cell infiltration.

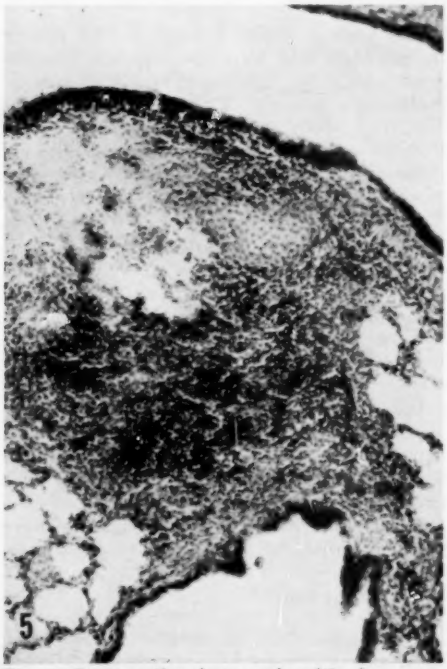

Fig. 5—Lung section from a pig with virus pneumonia of pigs, on the eleventh day after infection, showing an area of lymphoid hyperplasia between two bronchi. x 125.

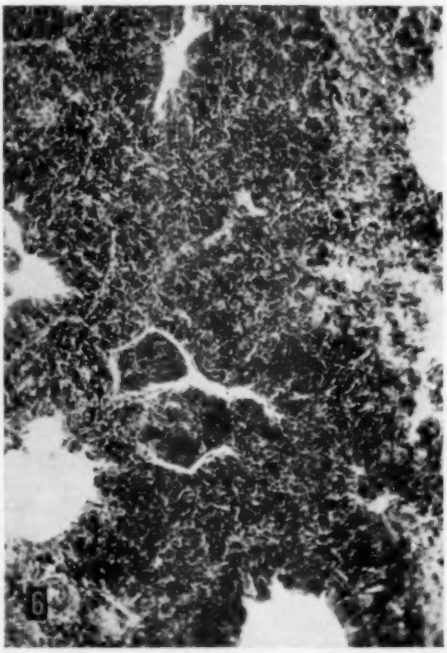

Fig. 6—Lung section from a pig with virus pneumonia of pigs, on the twenty-first day after infection, showing heavy round cell infiltration and proliferation around the bronchi. x 125.

A possible clue to why specific antibody for swine influenza virus is stimulated, whereas no antibody response is associated with VPP infection, is offered in these studies. Necrosis of tissue by action of the influenza virus may liberate antigenic substances which stimulate an antibody response. By contrast, VPP elicits a mechanical invasion of small and large round cells. Since there is no necrosis of host cells, enough antigenic materials may not be released to stimulate antibody formation.

SUMMARY

Disease-free, antibody-devoid pigs, obtained by hysterectomy and housed in isolation units, were infected experimentally when 1 week old with the viruses causing swine influenza or virus pneumonia of pigs

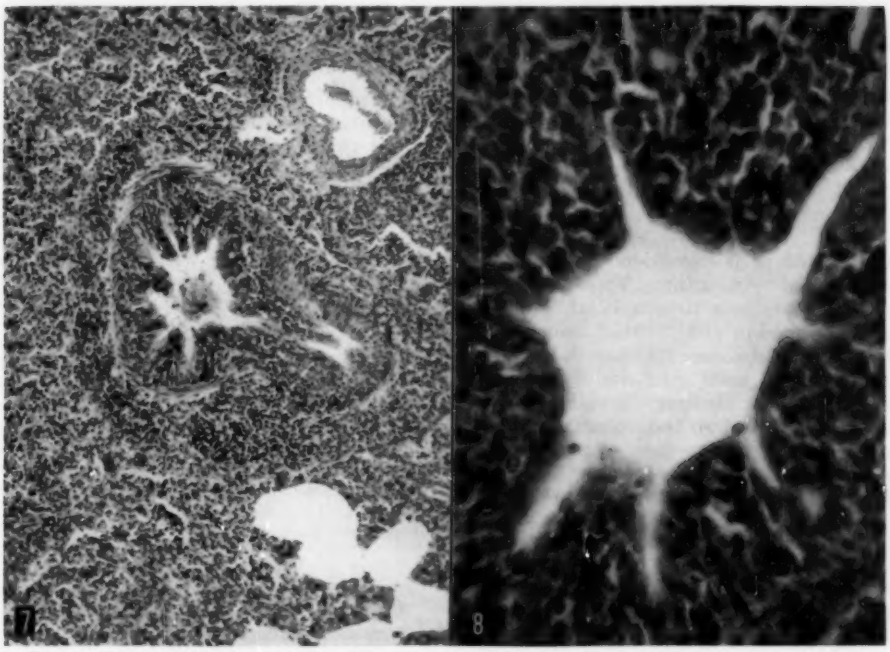

Fig. 7—Lung section from a pig with virus pneumonia of pigs, on the sixteenth day after infection, showing alveolar cell pneumonia. x 125.

Fig. 8—Lung section from a pig with virus pneumonia of pigs, on the nineteenth day after infection, showing lymphocytic (round cell) infiltration within the propria mucosa of a bronchus. x 400.

(VPP). Both viruses stimulate similar gross pathological changes consisting of plum-colored consolidation of the apical and cardiac lobes of the lung; however, the response of the bronchial lymph nodes was different. Histopathological changes in both diseases consist primarily of small and large round cell infiltrations.

Swine influenza virus elicited consistent tissue responses from the third through the fifth day which were distinct from VPP. Degenerative and regenerative changes were observed in this early period. From the sixth day on, however, small and large round cells were increasingly evident in perivascular and peribronchiolar reactions. Maximum cellular reaction had occurred by the eleventh day.

No histopathological changes were observed in VPP-infected pigs in the first ten days. After that time, a gradual but increasingly severe small round and lymphocytic cell infiltration occurred. Perivascular reactions were often so severe that it was difficult to distinguish the vessels. The lumina of bronchi were almost obstructed, due to hyperplasia of lymphatic tissue within the propria mucosa. Swine influenza virus did not elicit as marked an infiltration of round cells as VPP. There also was no evidence in VPP of the necrosis or regeneration characteristic of swine influenza virus infection.

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Cross Transmission of Nematodes of Domestic Animals
I. Experimental Infection of Swine with *Trichostrongylus Colubriformis*

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ALTHOUGH it is considered primarily a parasite of ruminants, *Trichostrongylus colubriformis* is a nematode of wide host range. Skrjabin *et al.*[5] listed domestic and wild ruminants, rodents, horses, monkeys, and man as hosts. In addition, Kotlán and Mócsy,[3] in Hungary, and Roberts,[4] in Australia, reported the occurrence of *T. colubriformis* in swine. Ivanitskiĭ[2] reported a trichostrongyle from a pig in Russia, for which he proposed the name, *Trichostrongylus suis*. So far as we are able to determine, the genus Trichostrongylus has not been reported in swine in the United States and no work has been done on experimental infections in swine.

The objective of this paper is to describe the experimental infection in swine. This has been partially reported, in abstract, by the writers.[6]

MATERIALS AND METHODS

Infective larvae were obtained by culturing the feces of cattle, sheep, or swine with charcoal or sphagnum moss. Larvae were recovered from the cultures in a Baermann apparatus and either used immediately or stored in water in a refrigerator for one day to four weeks.

Twenty-four pigs of mixed breeding, from 26 to 116 days old when infected, were exposed to *T. colubriformis* by oral administration of 2,900 to 114,600 infective larvae in single doses. During the course of the infection, the animals were observed daily, except on week ends, and weighed weekly. Beginning on the seventeenth day after infection and continuing at irregular intervals thereafter, flotations were made of the feces with saturated magnesium sulfate solution. Occasionally, the Stoll dilution technique was used when large numbers of eggs were detected by flotation.

The pigs were killed at various intervals after infection, and the following procedures were used routinely at necropsy. Ligatures were placed on the small intestine near the pyloric and cecal valves, and the intestine was excised and opened along its entire length. Ingesta were removed by gentle agitation in a pan of water, and the mucosal surface was examined for gross lesions. Portions of the tissue were fixed in formalin and later sectioned and stained with hematoxylin and eosin. The mucosal surface of the remainder was washed and scraped to remove adherent mucus. The washings and scrapings then were added to the intestinal contents, and a total count of worms present was made by a standard dilution procedure. Nematodes were examined either alive in saline solution or in lactophenol solution after fixation in 70 per cent alcohol.

RESULTS

Clinical Observations.—Of the 24 pigs, 19 became infected, as shown by patency or recovery of worms at necropsy (table 1). Pigs 78 days old or older could not be infected, whereas 100 per cent of the pigs 48 days old or younger became infected. Diarrhea was observed seven to 21 days after infection in 13 of the pigs, and persisted in some for three to five days. Pig 1401 became constipated, and blood appeared in the feces on postinfection days 20 and 21.

The prepatent period varied from 18 to 27 days, and the longest observed duration of patency was 26 days in pig 1403, which was killed 49 days after infection. Peak egg production occurred 21 to 29 days after infection and then declined rapidly; the maximum number of eggs per gram was 1,600. A typical set of egg count data from one series is shown (table 2).

The effect of *T. colubriformis* on the rate of growth of the pigs was variable. In one series (pigs 1401–1408), the rate of growth was depressed and weight losses amounted to as much as 8 lb. in pig 1408. In another series (pigs 1441–1449), the rate of growth was not significantly affected.

Pigs 1407 and 1408 displayed sluggishness, rough coat, and anorexia, beginning during the third week after infection. Their

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condition gradually deteriorated until, in the fourth week, both appeared moribund. On postinfection day 26, pig 1408 was killed; however, pig 1407, which was retained for further observation, survived and its physical condition was improving when it was killed, nine days later.

Necropsy Findings.—The data (table 1) show that recovery of worms ranged from less than 1 per cent to over 68 per cent of the larvae given. With the exception of pigs 1324, 1482, and 1490, which were killed during the prepatent period, all the pigs which were infected harbored only sexually mature adult worms. As the infection progressed, there appeared to be some diminution in numbers of worms recovered, although there was individual variation, as illustrated by pig 1403, from which nearly 50 per cent of the larvae given were recovered as adults, 49 days after infection. As anticipated by decline in egg counts, about 80 per cent of the female worms recovered from animals killed during the later phases of patency had few or no eggs in their uteri.

Pathological changes which could be associated with the presence of *T. colubriformis* were relatively slight, and the small intestines of all infected animals necropsied presented essentially the same picture. Grossly, there was localized congestion, with some areas of punctate hemorrhage, chiefly on the longitudinal folds; other portions of the mucosa exhibited a diffuse reddening. Examination of tissue sections revealed a slight to moderate eosinophilia in the lamina propria of the villi. A marked congestion and extravasation of erythrocytes was observed in the longitudinal folds, corresponding to the punctate hemorrhages observed on gross examination. Sections of nematodes were seen in the lumen and among the epithelial cells covering the tips or bases of the villi.

Discussion

Andrews[1] studied experimental trichostrongylosis in sheep and goats, and a comparison of his findings with our study indicates some similarities between the syndrome observed in those ruminants and that seen in swine. Andrews[1] observed a profuse, continuous, and occasionally bloody diarrhea appearing 13 to 59 days after infection with single or multiple doses of infective larvae and usually persisting until the death of the host. The prepatent period ranged from 17 to 21 days, with maximum egg production occurring 21 to 81 days after infection. Diarrhea and maximum egg count appeared earliest in those animals given large single doses of larvae. At necropsy, a diffuse

TABLE 1—Patency, Duration of Infection, and Recovery of Trichostrongylus Colubriformis After Administration of Single Doses of Larvae to Swine of Different Ages

Pig (No.)	Age when infected (days)	No. larvae given	No. days after infection		No. worms recovered
			Patency	Necropsy	
1324	26	1,200,000 M	....	6	420*
1401	31	23,200 S	21	54	Neg.
1403	31	16,300 S	23	49	8,030
1404	39	52,000 S	27	53	Neg.
1405	39	31,200 S	19	28	20,700
1406	39	41,600 S	28	44	10
1407	39	72,800 S	28	35	490
1408	39	41,200 S	19	26	6,140
1482	42	28,500 S	....	14	19,400†
1487	42	28,500 S	25	57	Neg.
1490	42	28,500 S	....	9	4,640**
1312	46	1,200,000 M	20	21	1,016
1441	46	30,900 S	20	51	Neg.
1443	46	30,900 S	20	83	Neg.
1445	46	30,900 S	19	83	Neg.
1446	46	30,900 S	20	29	11,130
1447	46	30,900 S	22	36	680
1448	46	30,900 S	21	51	Neg.
1449	46	114,600 S	20	78	Neg.
1442	48	2,860 P	20	28	440
1303	78	125,000 M	Neg.	20	Neg.
1304	78	35,000 M	Neg.	20	Neg.
1305	78	100,000 M	Neg.	55	Neg.
1079	116	14,000 S	Neg.	36	Neg.

* Larvae (parasitic third-stage and third molt); † sexually immature adults; ** larvae (fourth molt).
M = mixture of infective larvae of cattle origin, about 1 per cent *T. colubriformis*; S = sheep origin; P = swine origin.

TABLE 2—Egg Counts Per Gram of Feces of Swine Infected with Trichostrongylus Colubriformis

Pig (No.)	Date and egg count per gram									
	7/30	8/2	8/5	8/6	8/7	8/8	8/9	8/12	8/13	8/14
1401*	2	2	2	6	20	39	19	6	1	1
1403*	0	1	6	6	82	59	58	13	16	22
1404	0	0	0	0	0	0	0	0	2	1
1405	0	0	14	36	202	1,200	1,600	1,000	22	13
1406	0	0	0	0	0	0	0	0	0	1
1407	0	0	0	0	0	1	1	0	0	2
1408	0	0	6	17	190	133	89	7	Killed	....

* Infected July 9; remainder infected July 17.

enteritis without extensive lesions was observed in the small intestine. Worm recoveries varied from more than 69 per cent in fatal cases to less than 1 per cent in nonfatal cases, which had longer courses.

In our study, the diarrhea appeared earlier and was of shorter duration than was reported in sheep [1] and in only 1 animal was blood observed in the feces. Patency occurred later in swine and was of shorter duration than in sheep. Maximum egg counts were detected earlier but declined rapidly thereafter, as reported for sheep. The percentage of recovery of worms and the pathological changes observed in the small intestine were similar to those observed for ruminants.

These observations on swine appear to be in conflict with the generalization that parasites, if they become established in abnormal hosts, tend to exhibit a greater pathogenicity than in the normal host. *Trichostrongylus colubriformis* appeared to be well adapted to young swine, the host-parasite relationship differing only in degree from that observed by Andrews [1] in the normal ruminant hosts.

From the data presented, it would appear that age is the most important factor in infection of swine with *T. colubriformis*. Since there was no opportunity for prior infection, it must be concluded that the resistance to infection demonstrated by older pigs was an expression of age immunity rather than acquired immunity. Whether this immunity in older swine was the result of greater ability to produce antibody, or the fuller development and functioning of the reticuloendothelial system, or simply a modification of the physiological environment cannot be evaluated without further investigation.

SUMMARY

Experimental trichostrongylosis was studied in 24 pigs of mixed breeding, ranging from 26 to 116 days old. Infective larvae of *Trichostrongylus colubriformis* were administered *per os* in single doses of 2,900 to 114,600. When 19 pigs, 26 to 48 days old, were exposed, they became infected; the remainder, 78 to 116 days old, did not. The infection in swine was transitory, and pathological changes produced were minor. Clinical data and necropsy findings, when compared with Andrews' data [1] on trichostrongylosis in sheep and goats, showed similarities, differing mainly in degree.

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Essential Fatty Acid Deficiency—Its Role in Parakeratosis

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PARAKERATOSIS in swine is characterized by erythema, seborrhea and hyperkeratosis of the skin, and retardation of growth. A localized skin infection frequently is associated with the disease in swine.

It was first reported as a distinct entity and called "parakeratosis" by Kernkamp and Ferrin,[17] in 1953. Numerous reports on the clinical syndrome, pathology, etiology, treatment, and prevention have since appeared.[14, 15, 18-20, 22]

Parakeratosis is most prevalent in swine 7 to 12 weeks old. This period corresponds with one of the periods of most rapid growth. However, parakeratosis does occur outside these age limits. It has been diagnosed in pigs 4 to 5 months old and in pigs still nursing.

The disease is found in rapidly growing pigs under good management practices. The ration usually is considered good to excellent, and frequently is well fortified and balanced for all known nutrients. Antibiotics usually have been added as a growth stimulant. It is not unusual for an owner to describe his herd, before the onset of parakeratosis, as one of the best he has had.

The association of a deficiency of essential fatty acids with changes in the skin of simple-stomached animals, such as the rat and dog, have been known[2, 3, 9, 10, 21] for many years. It is apparent that the dermatosis of swine known as "parakeratosis" is, in many respects, similar to the disease entities found in the rat and dog. The essential fatty acids are considered necessary dietary nutrients because the rates of biosynthesis are not sufficient to meet the body requirements at certain times. In general, fats have not received as much attention from the swine nutritionist as have some other nutrients because they can be produced from carbohydrates and proteins. Today, most swine rations are low in fat.

During the last two decades, swine geneticists and nutritionists have worked toward increasing rates of growth, which has been accomplished through proper selection, cross breeding, and the development of new breeds of swine, and by feeding rations fortified with vitamin B_{12}, antibiotics, and high levels of proteins, and including almost every trace mineral and vitamin. The increases in rates of growth may have caused a concomitant increase in dietary requirements for essential fatty acids.

Greasy pig disease is another dermatosis of swine. It appears to be similar to parakeratosis in its epizootic characteristics and microscopic lesions. There also is some evidence that it responds to essential fatty acids.[13] It primarily affects pigs 1 to 5 weeks old. Jones,[16] in 1956, called this disease "exudative epidermitis," and reported the microscopic lesions associated with it.

The purpose of our study was to attempt to clarify the etiological factors involved in parakeratosis. In the past, repeated attempts have been made to isolate an infectious organism,[18] but they have not been successful. The similarities between essential fatty acid deficiencies in the rat and dog and this dermatosis in swine indicated that the metabolic aspects of the disease should be explored further. A study was undertaken to determine if a deficiency of unsaturated fatty acids was associated with the cause of parakeratosis.

REVIEW OF LITERATURE

The lesions due to unsaturated fatty acid deficiency in the rat and dog have received extensive and continued attention. The significant changes which occur in the rat on a fat-deficient ration are scaly skin, caudal necrosis, and retardation of growth.

Sinclair[21] reported the following picture of fat deficiency: The skin of the rat is affected over the entire body, at one stage or another. The malpighian and granular layers of the epidermis are

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increased in size, with pronounced hyperkeratosis. The keratin is collected into globules, with parakeratosis being evident. The keratotic lamellae are close together and lie in direct contact with the stratum granulosum. This acanthosis and hyperkeratosis also affect the lining epithelium of the opening of hair follicles, which become plugged. In the dermis, the capillaries are increased and dilated, with a mononuclear cell infiltration of the area. The sebaceous glands are intact and hypertrophied. This condition, if continued with the orifices of the hair follicles plugged, would eventually result in degenerative changes of the sebaceous glands. The dry appearance of the skin, which is characteristic of this condition in rats, is probably due to an obstruction of the outflow of sebum.

In fat deficiency, the rat at first gains weight; then the weight becomes constant and, finally, as the deficiency continues, the rat loses weight. This cessation of growth is probably caused by a shortage of essential fatty acid or acids available in sufficient amounts for the formation of phospholipids of new cell membranes.[22]

The fat-deficient rat responds favorably to linoleic acid and arachidonic acid in growth, improvement of skin lesions, and in decreased water consumption. Linolenic acid gives a less favorable response, which is centered almost entirely in a growth response and is not effective in correcting the skin lesions. Arachidonic acid is the most active of the three, and linoleic acid gives rise to arachidonic acid in the animal body, possibly explaining the more potent nature of arachidonic acid.[24] These acids appear to be necessary for vital architecture and the production of mobile lipids. No other fatty acids have shown curative action in this syndrome.

In the fatty acid deficiency syndrome, infections of the skin are common. The cause of death in dogs with fatty acid deficiency usually is considered to be infections because of decreased resistance.[12] In dogs, a continuous purulent discharge from the ears is seen under experimental conditions.[13] The bactericidal action of the skin of the rat and of the dog probably is entirely dependent upon the long-chain fatty acids and their soaps; the unsaturated fatty acids have a particularly high antiseptic action.[21] Skin infections might, therefore, be expected in fatty acid deficiency if the concentration of unsaturated fatty acids in the body is lowered.

The skin changes seen in the young dog on a ration low in fat (1% of the total calories) are dryness of the hair and skin, with flaky desquamation occurring.[10] At a fat level of 6 per cent of the total calories, there was marked improvement, but permanent and complete recoveries required levels of at least 16 per cent of the calories.

Microscopic examination of the skin of dogs deprived of dietary fat shows definite differences from the skin of the control dogs given a source of fat in their rations.[11] Hansen *et al.*[9] describe changes in the epidermis and dermis of the fat-deficient dog. These changes are a compact stratum corneum, which may show parakeratosis, and a thickening of the epidermis, with palisade and peg

TABLE 1—Rations Used in Parakeratosis Studies

First laboratory trial		Second laboratory trial			
Ingredient	Quantity	Ingredient	Quantity	Ingredient	Quantity
BASAL RATION (LOTS 1, 4, 6)		BASAL RATION (LOTS 12, 23)		LOW-ENERGY RATION (LOTS 16, 24)	
Bran	100.0 lb.	Bran	100.0 lb.	Yellow corn	35.0 lb.
Yellow corn	500.0 lb.	Yellow corn	500.0 lb.	Oats	25.0 lb.
Oats	150.0 lb.	Oats	150.0 lb.	Soybean oil meal (solvent process)	15.0 lb.
Tankage	75.0 lb.	Tankage	75.0 lb.	Salt	0.5 lb.
Soybean oil meal (solvent process)	150.0 lb.	Soybean oil meal (solvent process)	150.0 lb.		
Bone meal	35.0 lb.	Salt	5.0 lb.		
Salt	5.0 lb.	Vitamins A and D*	12.5 lb.		
Vitamins A and D*	12.5 lb.	Chlorotetracycline	2.0 lb.		
Chlorotetracycline	2.0 lb.				
Totals	1,029.5 lb.		994.5 lb.		100.5 lb.
		CALCULATED ANALYSES†			
Protein	18.9 %	Protein	18.9 %	Protein	16.0 %
Calcium	1.5 %	Calcium	0.7 %	Calcium	0.4 %
Phosphorus	1.0 %	Phosphorus	0.6 %	Phosphorus	0.3 %
Sodium chloride	0.5 %	Sodium chloride	0.5 %	Sodium chloride	0.5 %
Antibiotics	2 Gm./100 lb.	Antibiotics	2 Gm./100 lb.		
EXPERIMENTAL RATION		EXPERIMENTAL RATIONS		EXPERIMENTAL RATIONS	
Lots 2, 3, 5—Basal ration plus soybean oil at rate of 10%, first two weeks, and 18%, last two weeks.		Lots 13, 21—Basal ration plus soybean oil at rate of 23% for entire feeding period.		Lots 11, 22—Basal ration plus zinc sulphate at 200 p.p.m.	
		Lot 14—Basal ration plus calcium gluconate (20 cc. of 6% solution subcutaneously/day).		Lot 15—Basal ration plus hydrogenated fat (Hydrofol Glycerides T-57N) at rate of 16%.	

* Vitamin A, 110,000 U.S.P. units per pound; vitamin D_2, 200,000 U.S.P. units per pound; vitamin D_3, 50,000 U.S.P. units per pound. † Method described in Feeds and Feeding. By Morrison, F. B. 20th ed. Morrison Publishing Co., Ithaca, N.Y. (1944): 954–999.

formation. The dermis shows extensive edema, fragmented collagen, swollen endothelial cells with small lumina, hyperkeratinization of the hair follicles, plugging of the hair shafts, and enlarged sebaceous glands. After prolonged fatty acid deficiency, the sebaceous glands appear shrunken.

Kernkamp and Ferrin,[17] in 1953, and Kernkamp *et al.*,[18] in 1955, stated that parakeratosis is characterized by hard, dry, crusted proliferations of the superficial layer of the epidermis. Parakeratosis was classified as a benign disease affecting young swine between 8 and 16 weeks of age and usually terminating, spontaneously, in complete recovery.

The earliest detectable lesion of parakeratosis is the presence of red papules on the underside of the abdomen and flank areas of the pig. Seborrhea also is present; the tips of the papules may or may not be covered by keratin. Shortly thereafter, keratin begins to build up in the regions of the flank, pastern, fetlock, and hock. The nasal, periorbital, and aural regions of the head show similar lesions early in the disease. The disease may or may not continue to progress until the entire body is involved, with the skin heavily encrusted and with deep fissures in the wrinkled skin.

Skin infections are common and large abscesses are seen, especially on the hindlegs at a level above the hocks. In mild cases of parakeratosis, the appetite remains good and affected pigs continue to make satisfactory weight gains. In severe cases, marked depression occurs, the appetite decreases and the rate of gain is reduced. The mortality is low, but the economic loss through weight losses and reduced gains is considerable. The clinical duration of this disease is in proportion to the degree of severity of the stunting and the skin lesions. Pigs placed on pasture have recovered sooner than those left in the drylot where the ration remains the same.

The histological picture is confined to changes in the skin. The crusted masses are composed of keratin and debris. Large numbers of nuclei are scattered throughout the stratum corneum, which may be regularly or irregularly arranged in layers. The rete pegs are increased in number and length, and the number of basal cells increases. All epidermal elements, except the stratum lucidum, seem to be increased. Small collections of granulocytes may be seen in the epidermis. No significant tissue alterations occur in the dermis, according to Kernkamp.[18]

Hvidsten *et al.*,[15] in 1955, made determinations of the essential fatty acids in blood serum of pigs with parakeratosis and normal pigs. They followed the method of Wiese and Hansen[25] for the analysis of unsaturated fatty acids, and concluded that low levels of essential fatty acids in the blood are not associated with the development of parakeratosis.

Mineral imbalances[19, 20] are known to have an important role in parakeratosis, and calcium has been shown to be important in the pathogenesis of parakeratosis. There is a direct relationship between high levels of calcium in the ration and in the increased incidence and severity of the disease. High levels of calcium, *i.e.*, up to 2 per cent, as seen in occasional field cases and in experimental trials, are not essential for the occurrence of the disease. Parakeratosis is seen in herds on a calculated calcium level of 0.6 to 0.7 per cent of the ration, which is the level recommended by the National Research Council.

Recently, the role of zinc has received considerable attention. Tucker and Salmon,[22] in 1955, concluded that parakeratosis was a disease caused by a zinc deficiency. Most frequently, it is suggested that levels of zinc (given as zinc salts) in the feed should be approximately 100 to 200 p.p.m. Dosages above and below these levels are not as effective.[19, 20]

MATERIALS AND METHODS

First Laboratory Trial.—Ten normal, cross-bred Chester White pigs, 7 weeks old, were obtained from one source and 16 pigs of mixed breeding were obtained from a herd where parakeratosis has been in progress for one month. Of these 16 pigs, 8 were considered normal and 8 were considered affected with parakeratosis. On the basis of their skin lesions, 2 pigs were representative of each of the following four stages or phases of the disease: (1) severe and generalized, (2) severe and confined to the legs, (3) mild and generalized, and (4) mild and confined to the legs. Zinc had been added to the ration of these pigs two weeks before the herd was investigated.

The 8 normal pigs from the diseased herd were placed in lots 1 and 2. The 8 pigs with parakeratosis were placed in lots 3 and 4, with 1 pig in each stage of the disease in each lot. These pigs were approximately 12 weeks old at the beginning of the experiment. The 10 normal cross-bred Chester White pigs were placed in lots 5 and 6. The basal rations used in this trial are shown (table 1).

Pigs in each lot were fed the same ration, except that for lots 2, 3, and 5 the ration was supplemented with soybean oil at a level of 10 per cent by weight for the first two weeks and then increased to 18 per cent for two weeks before the end of the experiment. This oil was a highly refined soybean oil with a saponification value of 190 minimum, an acid value of 0.3 maximum, iodine value of 129, and an analysis of 54 per cent linoleic acid (98% of the acids had a chain length of C_{18}).

Second Laboratory Trial.—Fifty pigs were used in the second trial, 20 of which had parakeratosis. The 30 normal cross-bred Chester White pigs, 6 to 8 weeks old, were purchased from one source and placed in six lots (11 through 16) of 5 pigs each. The 20 pigs with parakeratosis were placed in groups of 5 (lots 21 through 24). The basal rations used in this trial are shown (table 1).

The following rations were given to pigs in the six lots used for the experimental production of

parakeratosis: lot 11, basal ration plus zinc (as $ZnSo_4 \cdot 7H_2O$) at 200 p.p.m.; lot 12, basal ration; lot 13, basal ration plus 23 per cent by weight of the oil described previously; lot 14, basal ration and subcutaneous injections twice daily of 10 cc. of a 6 per cent solution of calcium gluconate; lot 15, basal ration plus 16 per cent hydrogenated fat (Hydrofol Glycerides T57N*) with an iodine value of 1 (maximum), a melting point of 59 to 64 C., saponification value of 193 to 198, and an acid value of 1 (maximum); and lot 16, a bulky, low-energy ration (table 1).

In conjunction with these lots, four additional lots (21, 22, 23, 24), each composed of 5 pigs with parakeratosis, were used in an attempt to evaluate the treatment of parakeratosis with the oil-supplemented ration, the zinc-supplemented ration, the low-energy ration, and the basal ration. Over one half of the pigs had field cases of parakeratosis in various stages of development; the others had cases of parakeratosis experimentally produced in the first laboratory trial. An attempt was made to equalize the lots as to the severity of the skin lesions. The pigs in lot 24 were fed the low-energy ration given to lot 16. Pigs in the other lots were fed the basal ration given to lots 11 through 15 and, in addition, pigs in lot 21 were given 23 per cent by weight of the oil described, and pigs in lot 22 were given 200 p.p.m. of zinc (as $ZnSo_4 \cdot 7H_2O$), while pigs in lot 23 served as controls.

* Hydrofol Glycerides is produced by Archer-Daniels-Midland Co., Minneapolis, Minn., from whom the following data were obtained: unsaturation, 1.0 per cent; C_{14} fatty acids, 0.2 per cent; C_{16} fatty acids, 28.6 per cent; and C_{18} fatty acids, 71.2 per cent.

RESULTS

FIRST LABORATORY TRIAL

In lots 1, 2, 3, and 4, the disease-aggravating and -precipitating nature of the basal ration was indicated by the decreased rates of growth (graph 1). Spontaneous recovery was apparent in all lots in the third week, as determined by rates of growth. (The most severely affected pigs were in lot 3.) The improvements of the skin lesions were not as dramatic and quick as were the improvements in appetite, vigor, and thriftiness.

The average leukocyte count for pigs in lot 3 was 25,200 per cubic millimeter, as compared with 33,700 per cubic millimeter for those in the nontreated group (lot 4). The hemoglobin, serum calcium, and serum phosphorus values were within the normal range for both lots.

Skin biopsies, taken at the end of the fourth week, from posterior to and above the hock in all pigs in lots 3 and 4, showed histological changes typical of parakeratosis. However, in lot 3 the lesions were minimal, while in lot 4 the hyperkeratosis, parakeratosis, acanthosis, hyperemia, and cellular infiltration were apparent.

During the third week of the experiment, 3 pigs in lot 5 (basal ration plus oil) developed erythema and minimum

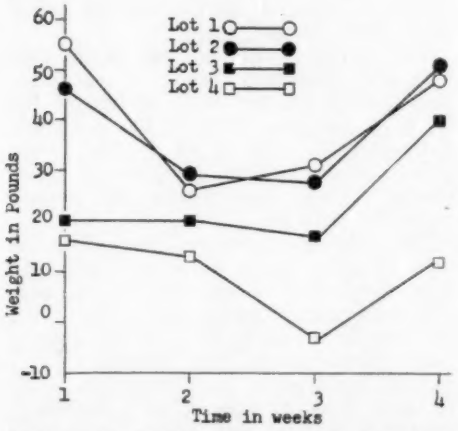
Fig. 1 and 2—A comparison of the gross lesions on the perinea of pigs in which parakeratosis was produced (lot 6) and the preventive effect of adding oil to the ration at 10 to 18 per cent by weight (lot 5).

amounts of keratinization of the skin of the perineum and hindlegs (fig. 1). The lesions were mild to moderate in 1, mild in another, and slight in the third. While clinical parakeratosis was not completely prevented, the disease was delayed in its onset and reduced in severity as compared with that in the control pigs in lot 6, where lesions occurred in all pigs starting on the ninth day (fig. 2, 3). The pigs in lot 5 did not show severe loss of weight or retardation of growth, as did the pigs in lot 6 (graph 2). The skin lesions continued to progress in all 5 pigs of lot 6 through the third week of the experiment, when 3 of the pigs were manifesting the severe, generalized form of the disease and 2 had severe lesions of the legs.

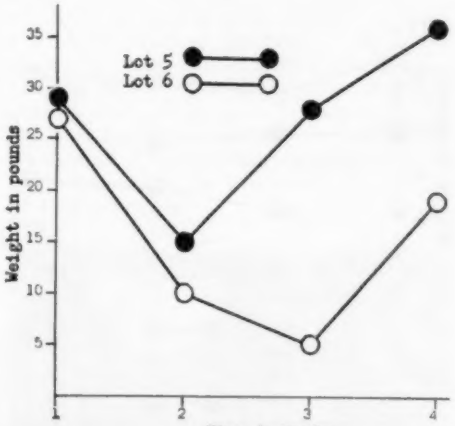
The stunting or decreased rates of growth became evident simultaneously with the onset of the skin lesions. In lot 6, 1 pig lost 2 lb. during the second week of the experiment and 4 pigs gained only 1 lb. each during the third week; each had gained nearly 6 lb. during the first week of the experiment. During the fourth week, the pigs in lot 6 started to show spontaneous recoveries (graph 2) without a change in the ration.

The lesions of the skin in experimentally produced parakeratosis follow a definite pattern. Initially, there is an erythema which may be generalized, covering the entire body, including the ears and the ventral abdominal wall and legs, or it may be confined to specific areas. In the early stages of the syndrome, almost without exception, slightly raised erythematous lesions appear first on the ventral abdominal wall and inner surface of the hindlimbs. The next most consistently involved areas are the ears and the orbital and postauricular areas. When the entire body and legs become erythemic, the disease usually progresses to a severe generalized form. Seborrhea occurs simultaneously with or immediately after the erythema.

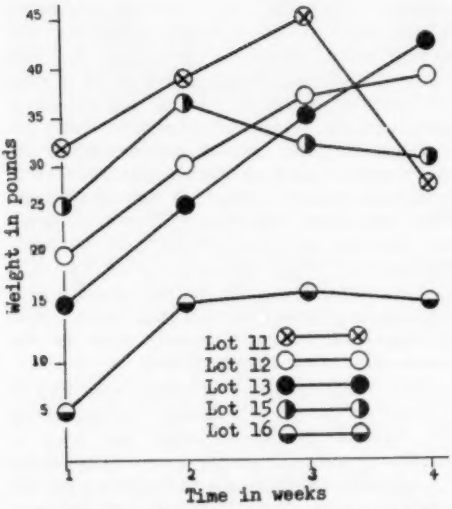
The distribution of the erythema may be diffuse or patchy. It usually is patchy on the ventral abdominal wall, but may be confluent over the entire area. Small areas of hyperkeratosis begin to build up on the erythematous skin; however, on the ventral abdominal wall the hyperkeratosis is minimal and does not build up progressively as it does in other areas. From the inner surface of the hindlimbs and ventral abdominal wall, the erythema and seborrhea extend dorsally and laterally to involve the skin of the perineum and the skin over the lateral surface of the hindlimbs. When this occurs, the ventral ab-



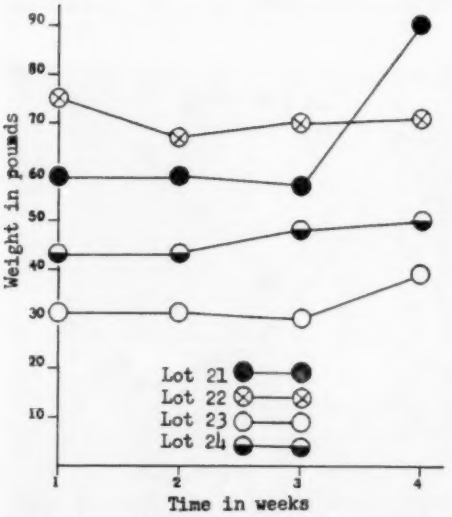
Graph 1—The weight gained per lot per week is shown for normal pigs (lots 1 and 2) from a herd with parakeratosis and pigs (lots 3 and 4) with parakeratosis (each lot contained 4 pigs). The therapeutic effect of oil on pigs with clinical parakeratosis, the disease-precipitating nature of the ration used, and the spontaneous recovery without a change in the ration are demonstrated. Rations fed each lot are shown in table 1.



Graph 2—The weight gains of pigs with experimentally produced parakeratosis (lot 6, 5 pigs) as compared with those of pigs (lot 5, 5 pigs) whose ration was supplemented with oil at 10 to 18 per cent by weight. The increase in weight gains without a change of ration demonstrates the spontaneous recovery associated with parakeratosis. Rations fed are shown in table 1.



Graph 3—Comparisons of weight gains made by pigs on a ration containing 0.6 per cent calcium by calculated analysis. There were 5 pigs in each of these lots, and the rations fed each lot are shown in table 1. Only lot 13, whose ration was supplemented with oil at 23 per cent by weight, failed to develop lesions associated with parakeratosis.



Graph 4—Weight gains of pigs with parakeratosis, which were treated by different rations. There were 5 pigs in each of these lots. Rations are listed in table 1. The growth-inhibitory effect of alfalfa meal is quite marked, as seen in lot 24. Lot 23 serves as a control.

dominal wall begins to clear up and will appear normal within seven to ten days, except for the peripheral areas along the hair line which still may be affected.

As the hyperkeratosis progresses, the skin becomes thickened and wrinkled, and fissures develop on the lateral abdominal walls and legs. Skin infection may be apparent grossly by the second or third week in moderate to severe cases. Small, circumscribed abscesses can be seen in the involved areas of the skin, and may vary in size from 1 to 3 cm. These abscesses are most apparent on the lateral surfaces of the hindlimbs, and usually contain a greenish purulent exudate. As soon as the skin changes occur, or shortly thereafter, the pigs show depression and listlessness, which increase as the skin lesions become more severe. The animal assumes a "humped" position, with the hindlegs drawn forward, and tends to spend more time in recumbency. The appetite usually remains good.

The rectal temperatures usually remain normal or near normal. Occasionally, there is moderate respiratory distress, with dyspnea and hyperpnea, in the severely affected pig. Lameness sometimes is seen in the early stages of the disease. When the abdominal lesions are developing, there usually is an increased periocular seborrhea and excessive lacrimation, the latter being constant and noticeable.

The average leukocyte count in the pigs with experimentally produced parakeratosis was 26,300 per cubic millimeter for lot 6, as compared with 23,100 per cubic millimeter for pigs in lot 5. The hemoglobin, serum calcium, and serum phosphorus values were within the normal range for both lots.

The histological changes observed in the skin biopsies were considered identical with those seen in skin biopsies of pigs with field cases of parakeratosis (fig. 5, 6). In those from pigs in lot 6, the hyperkeratosis, parakeratosis, peg formation, and cellular infiltration were extensive, while in those from the pigs in lot 5, 2 showed minimum changes and 3 were considered normal.

Second Laboratory Trial

In lots 11 through 16 (graph 3) where 30 normal pigs were used, all pigs developed skin lesions associated with parakeratosis except the 5 pigs in lot 13, which were

fed the basal ration supplemented with oil (23% by weight). All 5 pigs in lot 11 developed the initial ventral abdominal skin lesions by the fifth to the seventh day of the experiment. In 4 pigs, it progressed to the stage of moderate, generalized parakeratosis and, in the fifth pig, to a severe generalized condition. All showed some improvement or spontaneous recovery by the end of the experiment. In lots 12 and 15, the results were essentially the same as in lot 11, where the pigs were given zinc sulfate at 200 p.p.m. In all three lots, 4 of 5 pigs developed moderate lesions and the fifth developed severe, generalized lesions of parakeratosis. All 5 pigs in lot 13 (fed oil) remained normal and, at the end of the experiment, their hair and skin were bright, glossy, and smooth (fig. 4). The experiment with lot 14 could not be continued because, after one week, the tissue reaction from the injections of calcium caused depression and a decrease in food intake. In spite of this, all 5 pigs showed mild stages of parakeratosis. In lot 16, the rates of growth were markedly lowered, but mild lesions still developed. A comparison of the rates of growth that occurred in these lots during the experiment is shown (graph 3).

In lot 21 (oil supplement), the skin lesions stopped progressing after the fourth day of the experiment and, by the seventh day, the keratinous material started to fall off, exposing essentially normal skin. In lot 22 (zinc supplement), 2 pigs showed skin lesions which continued to progress slowly for two weeks, with increased keratin deposition, seborrhea, and thickening of the skin. This was followed by improvement, with the keratinous material sloughing. These pigs, however, were active, thrifty, and ate well. In lot 23, the skin lesions continued to progress rapidly, especially in 4 of the 5 pigs. Seborrhea, erythema, hyperkeratosis, depression, and dehydration were marked in 2 of the 4. In lot 24 (low-energy ration), there was little change in skin lesions during the first ten days. These pigs were not as severely affected as the pigs in lots 21 and 22; their appetites were good and they remained active and appeared thrifty. The keratin began to fall off during the tenth to twelfth days of the experiment. Their rates of growth were not spectacular, but their skin lesions improved as soon or nearly as soon as those of the pigs in lots 21 and 22.

Fig. 3—Pigs in lot 6, showing the gross lesions of experimentally produced parakeratosis.

The weight gained per week throughout the experiment for these four lots is shown (graph 4). Little difference in results could be observed when comparing the pigs on zinc and oil supplemented rations at the end of the experiment. The pigs fed the low-energy ration did not make the gains shown in lots 21 and 22, but did show marked improvement of their skin lesions.

Discussion

Early in the study, it was noticed that parakeratosis and greasy pig disease were similar in a number of ways. Both diseases were characterized by skin lesions, reduced rates of growth, and evidence of skin infection. In the corn belt states in the last decade, these diseases have been encountered as distinct clinical entities. A number of attempts to incriminate an infectious agent have failed. Both diseases occur under a variety of circumstances; they frequently are associated with rapidly growing pigs and good to excellent nutritional standards. These findings suggest a metabolic disorder as the probable cause of one or both diseases.

Fig. 4—No gross lesions of parakeratosis were demonstrable in pigs whose ration was supplemented with oil at 23 per cent by weight.

There has been considerable experimental investigation of all nutrients in swine rations. Ellis *et al.*,[5-7] among others, have reported on fat nutrition in swine. The swine rations used today, as well as those used in the past, are low in fat. The rations of pigs with parakeratosis and of the sows which farrowed pigs that developed parakeratosis frequently are well balanced and include some or all of the growth stimulants known to be required for swine. Calcium levels in these rations often are above those recommended by the swine nutritionist, and calcium is known to be associated with decreased lipid digestibility in the gastrointestinal tract. The spontaneous recovery seen in parakeratosis, which is especially apparent in the mild or moderately affected pigs, tended to indicate further that it is a metabolic disease.

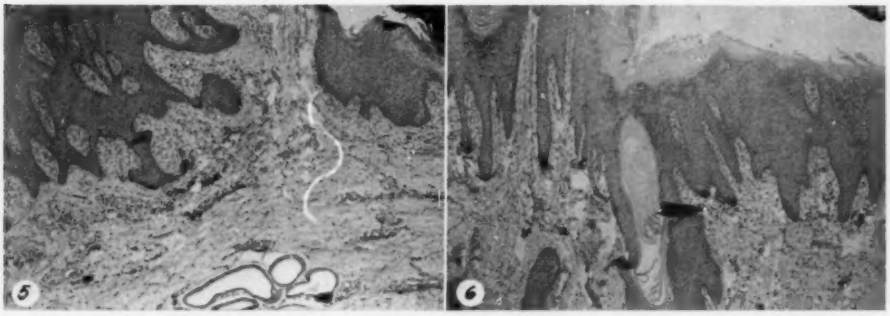


Fig. 5 and 6—The similarity of the microsopic changes in experimental (fig. 5) and clinical (fig. 6) parakeratosis is shown. H & E stain; x 37.

These findings presented a picture of rapidly growing swine on a low fat ration which is made even lower by excessive levels of calcium. Further, the lesions seen in these diseases were essentially skin alterations and reduced growth rates, which are comparable to the skin alterations and reduced rates of growth of the rat and dog with essential fatty acid deficiency. These findings implicated the essential fatty acids as the probable deficient nutrient causing both of these dermatoses.

Essential fatty acid deficiencies have been reproduced in the rat by Burr *et al.*[2-4] and in the dog by Hansen *et al.*[11,12] Some of the specific changes seen in the skin of the essential fatty acid-deficient rat or dog, which are also seen in the pig with parakeratosis, are increased or thickened epidermis, parakeratosis, hyperkeratosis, acanthosis, edema, cellular infiltration, hyperemia, plugged hair follicles, and hypertrophied sebaceous glands in the early stages, and atrophied sebaceous glands if the condition continues with the hair follicles plugged.

Lipid metabolism in swine has received little attention. It has been assumed that, since fats can be synthesized by the animal from carbohydrate and protein, their inclusion in the diet was not necessary; however, the unsaturated fatty acids are exceptions. It appears that, during periods of extremely rapid growth, the biosynthesis of these unsaturated fatty acids can not keep up with the requirements of the animal body. Spontaneous recovery from an unsaturated fatty acid deficiency, which occurs when the biosynthesis does catch up with the body requirements, frequently is seen in both greasy pig disease and parakeratosis of swine.

Calcium and magnesium decrease the digestibility of lipids in the diet, especially those of high molecular weight. This decreased digestibility is considered the result of a direct linkage of calcium and fat which occurs in the gastrointestinal tract. High levels of calcium, as shown in the second laboratory trial, are not essential for the disease syndrome but tend to increase the severity of the disease.

An important consideration in the experimental reproduction of parakeratosis is the source of the weaned pigs. In general, the swine population has been nutritionally and genetically conditioned toward a borderline unsaturated fatty acid deficiency. This deficiency is seen more frequently where modern swine breeding and

feeding programs are coupled with sound management practices. A deficiency of essential fatty acids in the pig is thought to be created by stimulation of the rates of growth, by breeding, balanced rations, and the addition of known growth stimulants to the rations, thus increasing the requirements of essential fatty acids over and above the rates of their biosynthesis.

In the first laboratory trial, the stunting, the severity of the lesions, and the short incubation or developmental period showed that the ration used was highly provocative. This was due to the extremes in the growth stimulants and to the high level of calcium. A provocative ration, rather than the duplication of rations found in the field investigations, was used in the first laboratory trial to insure reproduction of the disease. It also could be concluded that these pigs were in a state of borderline deficiency, with respect to unsaturated fatty acids, at the beginning of the experiment.

In the second laboratory trial, the pigs fed the basal ration alone (lot 12) developed parakeratosis, as did the pigs (lot 15) which were given the basal ration plus hydrogenated fat to determine if fat, per se, would prevent the disease. Also, pigs in lot 11 were given the basal ration plus zinc to evaluate the role credited to zinc by Tucker and Salmon.[22] The addition of zinc failed to prevent parakeratosis; however, parakeratosis did not develop when oil was added to the basal ration (lot 13). Lot 14 was included in an attempt to determine if calcium acted on lipid metabolism in some manner other than the direct linkage which is known to occur in the gastrointestinal tract; however, this attempt failed because of the depression and diminished appetite of these animals after the calcium injections.

In the first laboratory trial, the prevention of parakeratosis by supplementing the ration with soybean oil at an initial level of 10 per cent by weight was not completely successful. It was postulated that the level of oil in the first laboratory trial was not high enough to counteract the high level of calcium in the ration and still supply sufficient fatty acids. In dogs, the level of oil required to prevent the deficiency was 29 per cent of the total calories in the diet.[11] In the second laboratory trial, the level of calcium was lowered to the accepted level to demonstrate that high levels of calcium are not essential for the production of the disease. However, it is significant as one of the etiological factors in naturally occurring parakeratosis.

Zinc should still be considered the preferred treatment for parakeratosis, primarily because of its low cost. On the basis of the disappearance of the skin lesions and the improved appetites and rates of growth, little difference was noticed between the pigs with parakeratosis which were treated with zinc or with oil. In addition to the zinc supplement, it appears that a ration for the treatment of pigs with parakeratosis should contain alfalfa meal if the pigs are not on pasture. The growth stimulants should be lowered and the ration should include corn and oats. The calcium level should be lowered to 0.5 to 0.6 per cent. A level of calcium of 0.5 to 0.7 per cent usually can be attained without the addition of minerals when a balanced ration is used. The balanced ration used in the second laboratory trial contained no added minerals, yet it met the calcium requirements recommended by the National Research Council for growing swine. In a limited number of field trials, a ration of half corn and half oats, with 10 per cent alfalfa meal, 0.5 per cent salt, and 100 p.p.m. of zinc sulfate, had considerable therapeutic value for herd treatment. In addition, supplementing the ration with soybean oil for the severely affected pigs appeared justified.

Summary and Conclusions

Literature regarding unsaturated fatty acid deficiencies in the rat and in the dog are cited to show the similarities of these deficiencies to parakeratosis in swine.

In two laboratory trials, parakeratosis was produced in normal, healthy, weaned feeder pigs. A deficiency of essential fatty acid was thought to be created by excessive levels of growth stimulants in a well-balanced swine ration, with the rate of biosynthesis of the unsaturated fatty acids presumably being unable to meet the demands of the body.

High levels of calcium were used in the ration in the first trial, and normal levels of calcium in the second trial, to decrease the digestibility of the fat in the ration. In several lots, the basal ration was supplemented with a soybean oil preparation containing 54 per cent linoleic acid. In the

second trial, no skin lesions developed in the 5 normal pigs given this oil in the ration at a level of 23 per cent by weight, while the 25 pigs in five other lots developed skin lesions associated with parakeratosis. Included in the second trial were two lots of pigs whose basal rations were supplemented with zinc sulfate at 200 p.p.m. and hydrogenated fat at 16 per cent by weight. In both lots, the pigs developed skin lesions as quickly and extensively as did those of the lot on the basal ration without any supplementation.

When treating pigs affected with parakeratosis, there was little difference in the results when the ration was supplemented with oil or zinc. In addition to adding zinc to the rations of swine with parakeratosis, the calcium level should be lowered to approximately 0.5 to 0.6 per cent, growth stimulants should be reduced, and ingredients such as oats and corn should be included.

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Relative Potency of Some Steroid Hormones and Related Compounds on Canine Uterine Growth

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ESTRIN INJECTIONS have been shown to augment uterine growth in immature rabbits [5, 10, 23] and in immature rats.[3, 6, 7, 22, 30] Moreover, estrone was found more potent than estradiol in producing growth of the rabbit uterus.[28]

The estrogenic activity of stilbestrol and its effect on the growth of the uterus were demonstrated in immature rats,[29] and in castrated adult rats.[6]

The male sex hormone, testosterone, produced hypertrophy in the rat uterus.[17, 21] Corpus luteum extract had practically no effect on the growth of the uterus in immature rabbits [9] or rats,[5, 8, 14, 16, 27] unless the uterus had been brought into a state of maturity by estrin. It was also shown that an antagonism existed between estrogen and progesterone on the uterine growth of rats[1, 17, 19, 29] Uterine atrophy following castration was prevented by administration of estrogen in rabbits,[10, 15, 28, 25] in bitches,[26] and in monkeys.[16]

Augmented uterine growth in castrated rats was produced by estrogen and progesterone treatment. [7, 13, 18]

The cooperative activity on uterine growth of estrogen and androgen also was shown in castrated female rats.[11, 18, 20]

MATERIALS AND METHODS

Eighty-six mature and 58 immature bitches, 4 to 5 months old, were used. Of the 58 immature bitches, 31 were allotted to four groups, and those in three groups were injected with estradiol, testosterone, or stilbestrol, respectively, in a dose of 1 mg. daily for ten days. The fourth group was given stilbestrol for ten days, followed by 1 mg. of progesterone daily for five days. The synergistic effect of the various hormones on the uterine growth also was demonstrated on 38 ovariectomized adult bitches which were treated with estradiol for ten days, followed (except in 4) by progesterone, testosterone, ethisterone, pregnenolone, or desoxycorticosterone for five days.

Hormones and materials used were dissolved in oil and injected subcutaneously into the neck region in the required amounts. All treated bitches were killed on the day after the last injection and the uterus was dissected free, dried, and weighed. Control animals were killed and examined at the same time.

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RESULTS

When uterine growth was compared in the immature bitches, it was found that the controls had paler, more anemic, and relatively smaller uteri than the treated animals. The uteri in those injected with estradiol were hyperemic with enlarged blood vessels while, in those treated with testosterone, the uteri were filled with fluid and were heavier than in the other animals.

The group injected with stilbestrol alone showed excessive uterine development which exceeded that produced with other materials tested. The effect of stilbestrol on uterine growth was intensified by the administration of progesterone. The relative potency of these drugs on uterine growth in immature bitches is shown (table 1).

The uteri of 38 adult ovariectomized bitches were smaller and more anemic than those of the 48 entire control bitches. The administation of estradiol alone to ovariectomized bitches increased the uterine development and weight and enhanced its circulation. Bitches given estrogen followed by testosterone, progesterone, ethisterone, desoxycorticosterone, or pregnenolone developed uteri much larger and heavier than those of the 5 control ovariectomized animals (table 2).

DISCUSSION

Immature nontreated bitches possess small, pale, and anemic uteri, indicating that the ovary is inactive at this stage and does not secrete the hormones which activate the uterus and increase its development. The administration of hormones to such immature animals produced an activated and well-developed uterus, the effect differing with the hormone used. Thus, the

TABLE 1—The Comparative Relationship Between Uterine and Body Weight in Nontreated and Hormone-Treated Immature Bitches (Grouped According to Body Weight)

Exper. (No.)	Animals used	Average body weight (kg.)	Average uterine weight (Gm.)	Uterine weight / Body weight
1	27 control immature bitches.	3	0.39	13/10,000
2	8 immature bitches treated with 1 mg. of estradiol daily for 10 days.	4.37	1.67	30/10,000
3	8 immature bitches treated with 1 mg. of testosterone daily for 10 days.	4.5	2.40	51/10,000
4	7 immature bitches treated with 1 mg. of stilbestrol daily for 10 days.	4.5	3.45	75/10,000
5	8 immature bitches treated with 1 mg. of stilbestrol daily for 10 days, followed by 1 mg. of progesterone daily for 5 days.	4.75	3.90	80/10,000

administration of estrogen, estradiol, or stilbestrol increases the uterine development in immature bitches and activates intermittent uterine contractions which increase its circulation and metabolism. Apart from the direct action of estradiol on the uterus, it also may stimulate the secretion of pituitary gonadotrophins which may activate the ovary to secrete some ovarian hormones in amounts which might aid uterine growth. This may account for some immature graafian follicles which were noticed when the ovaries were examined. The estrogenic effect on the growth of the uterus, as shown in the bitch, correlated with results obtained in other animals by other investigators, who demonstrated an increased uterine weight by administration of natural and synthetic estrogens.[5-7, 10, 22, 23, 28, 30]

It was found that the synthetic estrogen, stilbestrol, was more potent in increasing canine uterine growth than the natural estrogen, estradiol. This may be due to the

TABLE 2—The Comparative Relationship Between Body Weight and Uterine Weight of Mature Bitches, Whether Entire or Ovariectomized, and Nontreated or Hormone-Treated (Grouped According to Their Body Weights)

Exper. (No.)	Animals used	Average body weight (kg.)	Average uterine weight (Gm.)	Uterine weight / Body weight
1	48 control nontreated bitches.	8.7	6.4	73/10,000
2	5 ovariectomized nontreated bitches.	6.3	4.7	66/10,000
3	4 ovariectomized bitches treated with 200 μg. of estradiol daily for 10 days.	10.5	17.1	160/10,000
4	10 ovariectomized bitches treated with 150 μg. of estradiol daily for 10 days, followed by 1 to 2 mg. of progesterone daily for 5 days.	8.4	6.4	76/10,000
5	5 ovariectomized bitches treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of testosterone daily for 5 days.	8.4	9.4	110/10,000
6	5 ovariectomized bitches treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of desoxycorticosterone daily for 5 days.	10.8	15.1	140/10,000
7	5 ovariectomized bitches treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of pregnenolone daily for 5 days.	7.2	11.4	160/10,000
8	4 ovariectomized bitches treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of ethisterone daily for 5 days.	8.0	8.25	110/10,000

more prolonged effect of stilbestrol, since estradiol is destroyed more quickly in the body. The effectiveness of repeated doses of stilbestrol was, therefore, considered to be considerably higher.[13, 17] It was found that stilbestrol is inactivated by the liver, but less rapidly than the natural estrogens.[21, 31]

The maximum growth effect on the uterus of the immature bitch was produced by stilbestrol, followed by progesterone. Apparently, stilbestrol acts on the uterus for a longer time, and acts synergistically with progesterone, increasing its progestational proliferating action on the endometrium. Also, both stilbestrol and progesterone may activate the pituitary to secrete some gonadotrophins which increase uterine development in the bitch through their action on the ovaries. This is in agreement with observations in rabbits[5, 8, 9, 14, 27] and in monkeys.[16]

To produce this synergistic effect, both estrogen and progesterone must be used in suitable quantities and in proper ratio; if not, antagonism might occur instead of synergism, as occurred in the mouse and guinea pig.[19]

Testosterone produced a greater enlargement of the uterus than did estradiol. This may be due to a direct specific action on the uterus, to conversion in the body of testosterone to estrogenic substance, to a synergistic action between testosterone and other hormones, or to some other relationship between the anterior pituitary, testosterone, and ovary. This needs further investigation.

The replacement therapy experiments on mature ovariectomized bitches showed that the steroid sex hormones and the related compound, stilbestrol, produced effects on the uterus similar to those obtained in immature bitches. In mature bitches, ovariectomy caused atrophy of the uterus, due to loss of the ovarian hormones that affect uterine growth.

Administration of estradiol to ovariectomized bitches caused hyperemia and intermittent uterine contraction in immature animals, thus increasing the flow of blood to and of lymph from the uterus and, consequently, increasing its metabolic rate and growth. The continued injections of estradiol for ten days produced a uterus more than twice as large as that of the controls (ratio 160:66). This finding is similar to that in rats,[1] in spayed rabbits,[5, 15, 25, 28] and in bitches.[26] The increase in uterine weight on administering progesterone, testosterone, ethisterone, pregnenolone, and desoxycorticosterone after priming with estradiol, in ovariectomized bitches, may be attributed to the synergistic action which exists between estrogen on one hand and progesterone and its similar substances on the other, as reported previously in rats with testosterone,[11, 18, 20] and with ethisterone.[12]

SUMMARY

1) The potency of the steroid sex hormones, estradiol, testosterone, and progesterone, and of the synthetic estrogen, stilbestrol, on uterine growth in immature bitches was investigated. All stimulated uterine development. Stilbestrol was most potent, especially when followed by progesterone; testosterone was second, and estradiol third.

2) Ovariectomized bitches given either estrogen alone or estrogen followed by testosterone, progesterone, ethisterone, desoxycorticosterone, and pregnenolone had much larger and heavier uteri than did untreated ovariectomized bitches.

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Effect of Adrenaline and Sex Hormones on the Uterine Motility of the Bitch

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A MYOMETRIAL MOTILITY CYCLE has been demonstrated by a number of investigators in the rabbit,[1, 12, 15, 16, 22] cat,[19] sow,[4] and buffalo.[20]

The suprarenal extracts caused strong contractions and increased frequency of the rabbit uterus.[2, 8, 9, 11, 21] Adrenaline inhibited the movements of gravid and nongravid guinea pig uteri.[5, 21] In the bitch and cat, adrenaline produced relaxation of the sterile uterus, whereas it produced vigorous contractions during pregnancy.[5, 6, 22] When a nonpregnant animal, castrated or not, was treated with estrogen or progesterone, adrenaline produced contraction instead of relaxation in castrated rabbits,[19] cats,[10] and in dogs and cats.[22] In the buffalo, adrenaline produced an increase in the frequency of uterine contractions and a decrease in their amplitude.[20]

In living animals, adrenaline produced uterine contractions in pregnant dogs,[18] in pregnant rabbits,[8] and in the intact gravid human uterus.[17]

The object of this work was to demonstrate, *in vitro* and *in vivo*, the action of adrenaline on the motility of the canine uterus at different stages of the sexual cycle. It was intended to investigate the responsiveness to adrenaline of uteri of ovariectomized dogs treated with various hormones.

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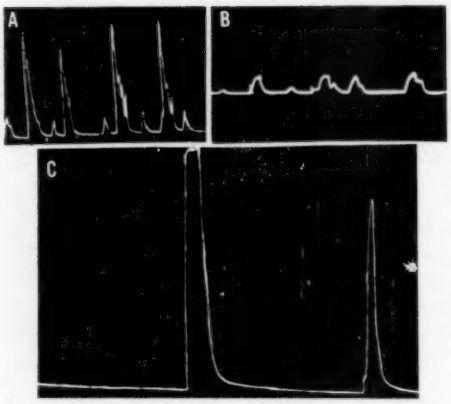
MATERIALS AND METHODS

Eighty-six bitches, 5 months to 4 years old, were used at different stages of their sexual cycles, or they were ovariectomized and treated with different hormones. The vaginal smear test was used to determine the stage of the sexual cycle, and pregnancy was determined by palpation, daily weighing, and laparotomy. Bitches to be treated with hormones were ovariectomized. After being examined or treated, the bitches were killed and a thin strip of their uterine muscle, about an inch long, was suspended in oxygenated Ringer's solution in the inner vessel (50-cc. capacity) of the bath at 37.5 C. When the normal rhythmic contractions of the uterus were recorded on smoked drum paper by a light lever at a low speed, adrenaline was added, in the required amounts, to the vessel containing the strip.

TABLE 1—In Vitro Motility of Uterine Muscle from Bitches at Various Stages of the Sexual Cycle and of Ovariectomized Bitches Treated with Different Hormones

Uteri	Motility
Immature bitch uterus	Small and few contractions
Immature bitch uterus treated with 1 mg. of estradiol daily for 10 days	Increased small contractions
Immature bitch uterus treated with testosterone, 1 mg. daily for 10 days	Small irregular contractions
Mature bitch uterus	
In estrus	Forceful rhythmic contractions
Nonestrous	Less forceful contractions
In pregnancy	Forceful contractions separated by long period of relaxation
Ovariectomized, nontreated bitch uterus	Decreased motility
Ovariectomized bitch uterus treated with 200 μg. of estradiol daily for 10 days	Increased uterine motility
Ovariectomized bitch uterus treated with 150 μg. of estradiol daily for 10 days followed by 1 mg. of progesterone daily for 5 days	Decreased uterine motility
Ovariectomized bitch uterus treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of testosterone daily for 5 days	Decreased uterine motility
Ovariectomized bitch uterus treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of ethisterone daily for 5 days	Increased uterine motility
Ovariectomized bitch uterus treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of pregnenolone daily for 5 days	Decreased uterine motility
Ovariectomized bitch uterus treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of desoxycorticosterone daily for 5 days	Increased uterine motility



Graph 1—Recordings of normal motility, in vitro, of uterine muscle of the bitch during various stages of the sexual cycle, showing motility (A) of an estrous bitch uterus, (B) of a nonestrous bitch uterus, and (C) of a pregnant bitch uterus.

To demonstrate the effect of adrenaline on the uteri of living bitches, a myograph described by Cushny [6] was used. This myograph contained two arms, arranged so that the short, movable one could respond freely to the uterine movements, which are recorded on smoked drum paper through a lever attached by a thread to the upper end and passing underneath a light pulley.

Animals were anesthetized by a mixture of 1 part alcohol, 2 parts chloroform, and 3 parts ether. Under anesthesia, the uterus was exposed through a midline incision and fixed with threads from two points (about 1 inch apart) onto the lower ends of the two arms of the myograph. The abdominal wall was stretched with threads to make a pouch in which warm Ringer and Locke's solution was added to keep the animal at normal temperature. All steroids used usually were dissolved in olive oil and injected subcutaneously in the neck region, using a fine needle. Adrenaline, supplied in ampules containing 1 mg. per milliliter was injected through the iliac vein and uterine artery.

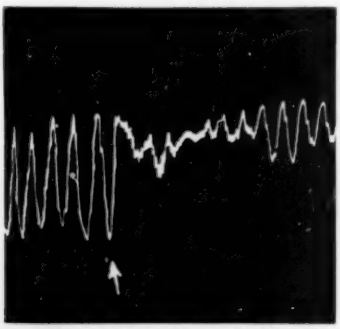
RESULTS

Forceful contractions were demonstrated *in vitro* in the uterine muscle of the bitch when in estrus, and smaller, less forceful contractions when not in estrus and when pregnant. The motility of uterine muscle from immature and ovariectomized bitches was sluggish and weak. The *in vitro* response to adrenaline of uterine muscle from ovariectomized bitches was increased when estradiol, alone or followed by desoxycorticosterone, was used, but was decreased when testosterone or pregnenolone were injected.

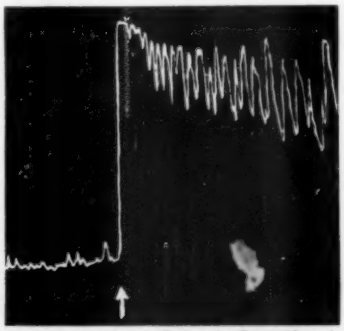
However, adrenaline inhibited uterine motility *in vivo* in the nonestrous bitch but

TABLE 2—In Vitro Effect of Adrenaline (1:500,000) on Uterine Muscle from Bitches at Different Stages of the Sexual Cycle and in Ovariectomized Bitches Treated with Different Hormones

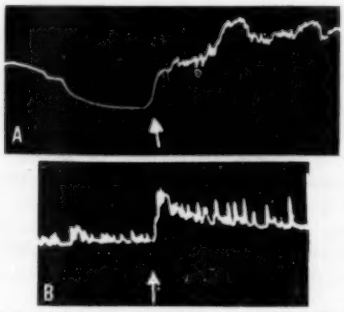
Uteri	Effect of adrenaline on uterine motility
Immature canine uterus	Inhibition
Nonestrous adult bitch uterus	Stimulation
Estrous bitch uterus	Stimulation
Early pregnant bitch uterus	Stimulation
Late pregnant bitch uterus	Stimulation
Ovariectomized bitch uterus	Inhibition
Ovariectomized bitch uterus treated with 200 μg. of estradiol daily for 10 days	Stimulation
Ovariectomized bitch uterus treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of testosterone daily for 5 days	Inhibition
Ovariectomized bitch uterus treated with 150 μg. of estradiol daily for 10 days, followed by 1 mg. of desoxycorticosterone daily for 5 days	Stimulation



Graph 2—The in vitro effect of adrenaline (arrow) on motility of uterine muscle from an estrous bitch.



Graph 3—The in vitro effect of adrenaline (arrow) on motility of uterine muscle from a nonestrous bitch.



Graph 4— The in vitro effect of adrenaline (arrows) on uterine muscle from (A) immature bitch treated with testosterone and (B) ovariectomized bitch treated with estradiol.

stimulated it in the estrous and pregnant animals.

Experiments on immature bitches showed that adrenaline inhibits uterine motility, but immature animals treated with estrogen and androgen showed motor uterine response. The results are shown (tables 1–3, graphs 1–7).

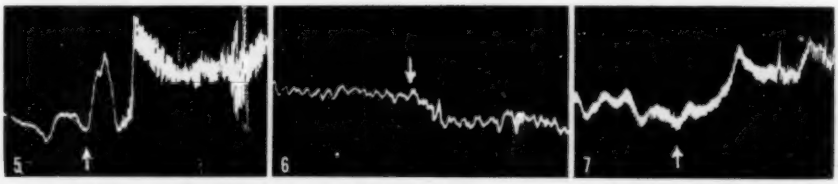
Discussion

The functioning of the excitatory and inhibitory fibers of the hypogastric nerve supplying the uterus of the bitch seemed to depend on the hormonal condition of the uterus. Thus, adrenaline stimulated the uterus in all conditions associated with the presence of estrogen, as during estrus and in ovariectomized animals treated with estradiol, and relaxed it in conditions where estrogens are not fully existing, as in immature and ovariectomized bitches. It also is probable that the presence of both estrogen and progesterone, as during pregnancy and in ovariectomized bitches treated with estradiol followed by progesterone-like substances such as desoxycorticosterone, will sensitize the uterus to the effect of adrenaline; hence, the increased uterine activity under these conditions. It seems possible that the hormones which increase the uterine motility and the response to adrenaline also may produce a direct action on the uterus by improving its circulation and increasing its metabolism and, consequently, an increased motility is obtained.

In ovariectomized bitches treated with estradiol, an increased uterine motility was noticed, whereas a decreased motility was demonstrated when progesterone and testosterone were injected after estradiol. It seems possible that 1 mg. of both progesterone and testosterone, given daily for five days, antagonized 150 μg. of estradiol given daily for ten days. Hence, an inhibition of the uterine movements was obtained when progesterone or testosterone were injected after estradiol treatment. Thus, when ethisterone, a progesterone-like substance, was injected in 1-mg. amounts daily, for five days following estradiol injections, and uterine movements were examined *in vitro*, an increased motility was noticed. This may be attributed to an inadequate dose of ethisterone to antagonize the stimulating effect of

TABLE 3—In Vivo Effect of Adrenaline on the Canine Uterus in Different Stages of the Sexual Cycle

Uteri	Effect of adrenaline	
	Intravenous	Intrauterine
Estrous bitch uterus	Stimulation	Strong stimulation
Nonestrous bitch uterus	Inhibition	Slight inhibition
Early pregnant uterus	Stimulation	Marked stimulation
Late pregnant uterus	Stimulation	Marked stimulation



Graph 5—The in vivo effect of adrenaline (arrow) on the motility of the uterus of an estrous bitch.

Graph 6—The in vivo effect of adrenaline (arrow) on the motility of the uterus of a nonestrous bitch.

Graph 7—The in vivo effect of adrenaline (arrow) on the motility of the uterus of a pregnant bitch.

estradiol, or ethisterone may be of an estrogenic nature, as reported for mice.[8]

Since uterine motility was increased in bitches treated with estradiol, followed by desoxycorticosterone, it seems that 1 mg. of this substance given daily for five days did not antagonize 150 μg. of estradiol given daily for ten days previously.

The fact that adrenaline stimulated the canine uterus in estrus and in pregnancy, both *in vivo* and *in vitro*, while it stimulated the nonestrous uterus *in vitro* but inhibited it *in vivo*, may be due to some change in the chemical structure of adrenaline in the body. Adrenaline action may depend, therefore, on the adrenaline products in the blood.

Since the effect of adrenaline on uterine motility *in vivo* intensified when it was given by intrauterine injection, it indicates that adrenaline, when given intravenously, is quickly destroyed in the body and produces little effect on the uterus. When injected directly into the uterine artery it acts strongly before being circulated extensively in the blood and destroyed.

In this work, adrenaline stimulated the intact gravid canine uterus, as it had previously in pregnant bitches [18] and in pregnant women.[19] In castrated bitches treated with estrogen, adrenaline *in vitro* excited the uterus and increased its frequency, as had been demonstrated in rabbits [21] and in cats.[10, 13]

However, *in vivo*, adrenaline stimulated the uterine muscle from estrous, nonestrous, and ovariectomized bitches treated with estradiol alone or followed by desoxycorticosterone, which seems to differ from the results of others,[5, 7, 21] who showed that adrenaline inhibited the nonpregnant uteri of cats and dogs.

SUMMARY

1) *In vitro*, adrenaline relaxed uterine tissue from bitches which were immature, ovariectomized, and ovariectomized and treated with estrogen followed by testosterone. However, adrenaline stimulated the motility of uterine tissue from immature bitches treated with estradiol or testosterone, from estrous, nonestrous, and pregnant bitches, and ovariectomized bitches treated with estradiol alone or followed by desoxycorticosterone.

2) *In vivo*, adrenaline stimulated the uteri of pregnant and estrous bitches, but inhibited the organ during nonestrous stages. Thus, *in vivo*, adrenaline inhibited the uterine movements during the nonestrous stages, while adrenaline stimulated the uterus *in vitro*.

3) Increased uterine motility was noticed in bitches in estrus, or which were ovariectomized and treated with estradiol. Motility was decreased in immature, nonestrous, and pregnant bitches, and in ovariectomized bitches treated with estradiol followed by progesterone or testosterone.

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A Study of the Anesthetic Properties of Combinations of Hexobarbital Sodium and Thialbarbitone Sodium in Dogs

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CARRINGTON AND RAVENTOS [2] reported that a new intravenous anesthetic, thialbarbitone sodium, (5-[2,3-cyclohexenyl]-5-allyl-2-thiobarbituric acid; Kemithal*), possessed properties that recommend it for canine surgery. In our hands, this agent did not give the desirable level of anesthesia for general use with an adequate margin of safety. However, thialbarbitone did possess two desirable properties of the ideal, extremely short acting, intravenous anesthetic, *i.e.*, smooth induction and an uncomplicated recovery.

Hexobarbital sodium (5-methyl-5-[1,2-cyclohexenyl]-N-methylbarbituric acid; Evipal†) was also found to be unsatisfactory as an extremely short acting intravenous anesthetic for dogs, for different reasons than for thialbarbitone; induction is not smooth and, more important, recovery is associated with a marked increase in excitability. Nevertheless, hexobarbital produces a good, controllable anesthesia. The data presented here were obtained during a study designed to determine whether any combination of these two barbiturates would possess, predominantly, the desirable properties of each agent, *i.e.*, the recovery properties of thialbarbitone and the anesthetic properties of hexobarbital.

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* Kemithal is produced by Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

† Evipal is produced by Winthrop Laboratories, Inc., New York, N.Y.

MATERIALS AND METHODS

Mongrel dogs of either sex, weighing between 5.5 and 24.7 kg., were used.

All injections were made intravenously, over a one-minute interval, as freshly prepared 5 per cent aqueous solutions of the sodium salts.

Graded responses were used to determine the degree of muscle relaxation and type of recovery (table 1). Any graded response less than three was not considered satisfactory.

RESULTS

The initial investigations were concerned with the anesthetic and recovery properties of hexobarbital and thialbarbitone. All doses of hexobarbital studied produced satisfactory muscular relaxation and, conversely, unsatisfactory recoveries (table 1). A dose of 35.4 mg. of hexobarbital per kilogram of body weight produced near maxi-

TABLE 1—Anesthetic Effect of Hexobarbital Sodium, Thialbarbitone Sodium, and Combinations of These Two Drugs in Dogs

Drug (Na salts)	Dose (mg./kg.)	No. Dogs	Av. jaw relaxation		Av. abdominal relaxation		Time to raise head (min. ± s.e.)	Time able to stand (min. ± s.e.)	Average recovery* (range)
			Degree* (range)	Duration (min. ± s.e.)	Degree* (range)	Duration (min. ± s.e.)			
Hexobarbital	25.0	4	3.0	18.8	3.5	18.8	25.0	45.0	1.0
Hexobarbital	35.4	9	3.7 (3.0–4.0)	30.2 ± 6.3	3.7 (2.0–4.0)	31.6 ± 5.9	75.5 ± 16.0	121.5 ± 24.0	1.2 (1.0–2.0)
Hexobarbital	50.0	4	4.0	61.8	4.0	61.8	119.0	.....	1.0
Hexobarbital–4, thialbarbitone–1	35.4	5	3.2	.....	3.0	.....	43.0	84.0	2.2
Hexobarbital–2.5, thialbarbitone–1	35.4	10	3.1	20.3 ± 4.2	3.4	19.8 ± 4.2	37.9 ± 6.0	65.3 ± 5.1	2.9
Hexobarbital–1, thialbarbitone–1	35.4	10	3.3 (2.0–4.0)	15.2 ± 1.9	3.9 (3.0–4.0)	17.4 ± 2.8	32.0 ± 3.9	54.0 ± 8.6	3.9 (3.0–4.0)
Thialbarbitone	35.4	8	1.7 (1.0–3.0)	7.8 ± 2.9	2.0 (1.0–3.0)	6.4 ± 2.5	23.3 ± 2.9	37.6 ± 4.8	4.0
Thialbarbitone	55.0	4	3.2	29.8	3.2	44.7	54.8	160.0	4.0

* Graded response: 1 = poor relaxation or unsatisfactory recovery; 2 = fair relaxation or recovery; 3 = good relaxation or recovery; 4 = excellent relaxation or recovery.

TABLE 2—Effect of Repeated Injections of a Combination of Equal Parts of Hexobarbital and Thialbarbitone on the Type of Recovery from Anesthesia in Dogs

Dog No., sex, weight (kg.)	First dose	Time able to stand (min.)	Recovery*	Second dose†	Time able to stand (min.)	Recovery
1810, M, 8.8	63.1 mg./kg.	136	4	31.6 mg./kg.	345	4
1920, M, 9.7	63.1 mg./kg.	195	4	31.6 mg./kg.	>420	4
1604, F, 7.6	63.1 mg./kg.	<300	4	31.6 mg./kg.	242	4
1832, F, 10.1	63.1 mg./kg.	200	4	31.6 mg./kg.	196	4
588, F, 11.3	63.1 mg./kg.	<300	4	31.6 mg./kg.	>276	4
1703, M, 11.8	63.1 mg./kg.	<300	4	31.6 mg./kg.	247	4

* Graded response (*see* table 1); † second dose given at about the time dogs were able to stand after the first dose.

mum relaxation and was selected as the standard for anesthetic comparison. Thialbarbitone did not produce adequate relaxation until 55 mg. per kilogram was used. Nevertheless, this barbiturate, at 35.4 mg. per kilogram, produced a highly desirable type of recovery. Since a combination exhibiting the anesthetic properties of hexobarbital and the recovery properties of thialbarbitone was desired, the 35 mg. per kilogram dose of the latter also was used as a standard for comparison. Only the combination of equal parts of the two agents exhibited the desirable actions of each. This combination also exhibited durations of action intermediate to those of the components.

The excellent recoveries mentioned were observed after the subjects were held at the anesthetic level for only a brief interval (approx. 15 min.). It was considered possible that the character of the recovery might be changed, should sufficient drug be administered to prolong the anesthesia. This was investigated in two ways: (1) A relatively large dose (63.1 mg./kg.) of the combination was given and, at the time the dogs were able to stand, an additional dose (31.6 mg./kg.) was given. (2) Sufficient amounts of the combination were given to maintain unquestionably excellent anesthesia for at least one hour. The desirable recovery was realized in both types of experiments (tables 2, 3). Although both barbiturates are classified as extremely short acting because of the rapid recovery after small doses (table 1), successive injections of the combination result in prolongation of narcosis.

The lethal dose for 50 per cent of the dogs ($l.d._{50}$) for hexobarbital, using 18 dogs, was found to be 90.8 ± 15.3 mg. per kilogram; for thialbarbitone, using 39 dogs, the $l.d._{50}$ was found to be 105.7 ± 9.8 mg. per kilogram. The observed $l.d._{50}$ for the combination, using 30 dogs, was found to be 93.7 mg. per kilogram and was not significantly different from the expected (calculated) $l.d._{50}$ of 98.0 mg. per kilogram. Thus, the toxic components of hexobarbital and thialbarbitone were considered additive; this conclusion is based on results obtained by using a method for the analysis of combinations described by Weaver *et al.*[3]

TABLE 3—Type of Recovery from Excellent Anesthesia for One Hour in Dogs Induced by a Combination of Equal Parts of Hexobarbital and Thialbarbitone

Dog No., sex, weight (kg.)	Doses (mg./kg.) in order of administration	Total dose (mg./kg.)	Recovery*	Time able to stand (hours)
2882, M, 9.3	35, 20, 20, 20	95	4	>7.5
2334, F, 7.8	35, 20, 20, 20	95	4	7.0
2669, F, 12.8	35, 20, 20, 20	95	4	>7.5
2908, F, 9.8	35, 20, 20, 20	95	4	>7.5
2101, F, 9.1	35, 20, 20	75	4	3.5

* Graded response (*see* table 1).

Discussion and Summary

A combination of equal parts of hexobarbital and thialbarbitone retains the desirable recovery characteristics of thialbarbitone, regardless of the length of anesthesia, and the degree of anesthesia appears to be at least additive. The duration of anesthesia is intermediate to that of each component.

Based on observations of the physiological disposition and chemical fate of hexobarbital and thialbarbitone in dogs, Brodie [1] concluded that these two barbiturates owe their apparent short action to the redistribution from plasma to fat. The fatty tissue ratio (tissue concentration: plasma concentration) three hours after the administration of 40 mg. per kilogram of each of these barbiturates to dogs was: thialbarbitone, 6.0, and hexobarbital, 3.2. Other tissues studied (liver, kidney, and muscle) did not show this difference in ratio. These data suggest that the predominant influence of thialbarbitone on recovery can not be explained by a nonselective redistribution of each barbiturate from plasma to fat. If this redistribution were the only factor concerned with the extremely short action of these agents, then the hexobarbital type of recovery would be expected. Thus, it would appear possible that, when these two barbiturates are present simultaneously, a selective redistribution of hexobarbital over thialbarbitone might be manifested. An alternate possibility is that additional mechanisms have a role in the brief action of at least hexobarbital.

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Electrophoretic Mobility of Canine Serum Maltase

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SEVERAL STUDIES [1-3,5] have shown that maltase occurs regularly in the serum of dogs. An earlier report from this laboratory [5] dealt with a method of estimating the activity of canine serum maltase and with certain properties of the enzyme which were of consequence in the development of this procedure. In connection with attempts to purify this enzyme, it became important to know with which fraction or fractions of the serum proteins it was associated. Filter paper electrophoresis was selected as the method to achieve this.

MATERIALS AND METHODS

For this study, fresh serum samples were obtained from 5 healthy, adult, male, mongrel dogs.

Canine serum (0.01 ml.) was applied by micropipette to Schleicher and Schuell S&S598 filter paper strips (3.5 by 37.5 cm.), the ends of which were immersed in barbital buffer (pH, 8.6; ionic strength, 0.075) within a hanging closed-strip paper electrophoresis cell. A constant current of 0.27 milliamperes (ma.) per centimeter of paper width was applied for a period of 20 hours at room temperature (21 to 23 C.).

Each serum sample utilized four filter paper strips. At the end of the run, either strips 1 and 3 or 2 and 4 were selected for detection of maltase activity. Immediately after their removal from the electrophoresis cell, these strips were cut across the long axis into 1-cm. segments. A total of 16 such segments were cut from each strip, beginning 3 cm. on the cathodal side of the point of application. The corresponding segments of each strip were then placed together in a tube with 8 ml. of 0.02 M phosphate buffer (Na_2HPO_4/KH_2PO_4), pH 6.6, which contained maltose at a given concentration. A total of five runs were made at a maltose concentration of 5.6×10^{-4} M, using serum from a different dog for each run. An additional two runs were made at a maltose concentration of 5.6×10^{-3} M, using serum from 2 of the same 5 dogs.

The tubes were shaken gently for approximately one minute to elute the protein from the strips and then were incubated for three hours in a constant temperature bath at 39 C. At the end of the incubation period, the reaction was stopped and a protein-free filtrate was obtained by adding to each tube, first, 1 ml. of 10 per cent $ZnSO_4$, followed by 1 ml. of 0.5 N NaOH, and then filtering. A blank containing the identical maltose-phosphate buffer solution but no segments from the filter paper strips was treated similarly.

The concentrations of the reducing substances in the filtrates were determined by the colorimetric method of Nelson [4] and, by comparison with the blank, the percentage of the maltose hydrolyzed in each tube was calculated.

The remaining two strips were oven-dried at 120 C. for one half hour and subsequently were stained for proteins with bromophenol blue. Analysis of these strips was done in an automatic recording and integrating densitometer.* The protein profiles of these two strips were compared to insure uniform protein migration in each run.

The protein profile of the stained strip which had been positioned between the unstained strips in the electrophoresis cell was used to locate the fractions possessing maltase activity. This was done by locating the 1-cm. segments on the stained strip which corresponded to the tubes containing segments from the unstained strips.

RESULTS

In all cases in which the initial maltose concentration was 5.6×10^{-4} M, the results demonstrated that maltase activity was associated exclusively with the *alpha*-1 and *alpha*-2 globulins. The major portion of this activity was associated with the leading edge of the *alpha*-2 globulin. The results of a typical run are illustrated (graph 1).

These experiments demonstrate only the electrophoretic properties of serum maltase. The results can not be used to establish an average value for the percentage of maltose hydrolyzed by the various segments, a value which might be used as an estimate of the amount of maltase present. Unfortunately, the length of the protein migration varies slightly from one run to another. For this reason, a greater or lesser proportion of a given protein fraction may be found in a particular segment in different runs. Maltase activity, being associated with the *alpha*-1 and *alpha*-2 globulins, necessarily varies in a given segment, depending on the distance which

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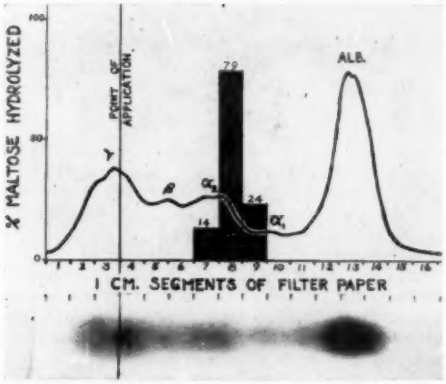
* The Spinco Analytrol, manufactured by Beckman Instruments, Inc., Spinco Division, Belmont, Calif.



Graph 1—Relationship of maltose hydrolysis to the protein profile. The histogram indicates the percentage of maltose hydrolyzed in tubes containing 1-cm. segments of electrophoresis strips (initial maltose concentration is 5.6×10^{-4}; run No. 2). Protein profile is superimposed. Stained strip is shown below.

these proteins migrate. Therefore, maltase activities of the segments of a particular run can be used to estimate the location and relative activities of the protein fractions of that run only.

In neither case in which the initial concentration of maltose was 5.6×10^{-3} M was hydrolysis of maltose demonstrable. This is in keeping with an earlier finding[5] which showed that, although dog serum is capable of hydrolyzing large amounts of maltose, sufficient elevation of the substrate concentration will block this activity completely.

SUMMARY

By the use of filter paper electrophoresis and subsequent elution of the proteins from the filter paper strips, it was demonstrated that canine serum maltase is associated exclusively with the *alpha*-1 and *alpha*-2 globulins, the major portion of the activity residing with the *alpha*-2 globulin. Further evidence that high concentrations of the substrate, maltose, will inhibit the activity of canine serum maltase was presented.

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The Effect of Antibiotics on Bacteria in Semen and on Motility and Fertilizing Ability of Chicken Spermatozoa

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IT IS COMMON PRACTICE in the storage of bull semen to add antibiotics in the diluent, usually streptomycin and penicillin. The extensive literature on this subject has been reviewed by Emmens and Blackshaw.[4] Since fecal contamination is more of a problem in chicken semen, it seems likely that the initial number of bacteria usually is greater than for bull semen. Failure to control bacterial growth is a possible explanation for poor results obtained in storing chicken semen. Smith,[7] working with chicken semen, found sulfathiazole but not streptomycin to be toxic at levels of 50, 100, and 200 μg. per milliliter. Although streptomycin checked bacterial growth at these levels, it did not improve fertility.

Since there is little information on the effect of antibiotics on chicken semen, experiments have been conducted to determine the effect of several of them on the fertilizing ability of chicken semen after storage. To reduce the number and levels of antibiotics used in fertility tests, the effect of several levels of the antibiotics on motility was checked first. Levels which proved to be toxic were not used in subsequent tests. Then the effect on bacterial count of different levels of antibiotics, and certain combinations, was tested. The two most promising antibiotics and levels in these tests were then tested further for effect on fertilizing ability.

EXPERIMENTAL PROCEDURE

The antibiotics selected were penicillin G sodium,* streptomycin sulfate,† dihydrostreptomycin sulfate,† chloramphenicol (Chloromycetin**), oleandomycin phosphate,‡ tetracycline hydrochloride (Tetracyn HCl‡), and oxytetracycline hydrochloride (Terramycin‡). The activity of all of these will be expressed in micrograms except for penicillin and chloramphenicol, which will be expressed in units.

Males of a flightless strain (90% New Hampshire and 10% White Leghorn) were used as the semen source. In fertility tests, males of a Barred Columbian strain were also used. White Leghorn females in individual batteries were used to test fertilizing ability. In all experiments, the semen was collected by a one-man technique described previously.[3] Dilutions were made by mixing pooled semen with an equal amount of diluent, followed by addition of enough diluent to give a dilution of 1:10. Diluted solutions were held at 10 C. The diluent used was a buffer containing 16.34 Gm. of Na_2HPO_4 and 5.16 Gm. of $NaH_2PO_4 \cdot H_2O$ per liter. In some motility experiments, this was diluted with an equal volume of a saline solution (9.9 Gm. of NaCl per liter). The antibiotics were added in the diluent; the stated levels are those in the diluted solution.

Motility was determined after storage for 24 and 120 hours. The solutions were held at room temperature for one hour before reading, a procedure which provides maximum motility. The classification of Smith[7] was used, in which scores ranged from 1 (no motility) to 4 (swirling).

In experiments on the bacterial number, the diluted semen was stored for 48 hours. Bacterial count was determined on semen just after collection, as well as on the diluted stored semen. The number of bacteria was determined, after incubation for three days at 37 C., aerobically by plate count on eugon agar (BBL) and eosin methylene blue agar, and anaerobically by turbidity in a thioglycollate broth culture. Bacterial counts were computed on the basis of the number in whole semen. Since there was a substantial difference in storage time from the plating of the first to the last sample, each experiment was done in replicate but, in the replication, the samples were plated in reverse order so as to correct for the difference in storage time.

In fertility tests, a procedure similar to that used previously[3] was followed, except that 0.1 ml. of the diluted semen was inseminated, instead of

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* The penicillin G sodium is produced by E. R. Squibb and Sons, Brooklyn, N.Y.; † the streptomycin sulfate and dihydrostreptomycin sulfate by Merck Sharp and Dohme, Philadelphia, Pa.; ** Chloromycetin by Parke Davis and Co., Detroit, Mich.; and ‡ the oleandomycin phosphate, Tetracyn HCl, and Terramycin by Chas. Pfizer and Co., Inc., Brooklyn, N.Y.

0.2 ml. Between 8 and 11 hens were used per treatment, except that only 6 were used in one treatment of trial 3. In the first trial, diluted semen was stored both anaerobically and aerobically. The former was accomplished by storage in a corked test tube filled completely with the diluted semen, the latter by using large test tubes with cotton in the tops. Since the fertility in this experiment was low and since it was not deemed desirable to appreciably shorten the storage time, in the next three trials the diluted semen was brought back to its original volume just before insemination. It was centrifuged at 1,200 R.C.F. (relative centrifugal force) for ten minutes, the supernatant fluid was poured off, and the packed cells were diluted to the original volume before dilution with the buffer. In this case, only aerobic conditions were used.

RESULTS AND DISCUSSION

Motility.—Motility scores are given (table 1). All antibiotics were first tested at 0.9, 9.0, 90.0, and 900.0 μg., or units, per milliliter; those toxic at 900 μg., or units, per milliliter also were tested at 180 and 450 μg., or units, per milliliter. For all antibiotics studied, there was no reduction in motility score after 24 hours' storage at levels of 0.9, 9.0, 90.0, and 180.0 μg., or units, per milliliter. In the case of oleandomycin, streptomycin, and penicillin, this was also true at 900 μg., or units, per milliliter, but there was a marked reduction in motility with oxytetracycline, tetracycline, and chloramphenicol at 900 μg., or units, per milliliter, and a moderate reduction in motility at 450 μg., or units, per milliliter. Motility was improved with oleandomycin, tetracycline, and oxytetracycline at one or more levels; however, there is insufficient replication to draw conclusions in this regard. Motility scores of semen stored 120 hours are not presented, since there was no difference between samples with and without antibiotics, except those in which the motility was adversely affected after storage for 24 hours.

The pH of the diluted solutions was recorded using a Beckman meter, model G, after 120 hours of storage. The pH of the semen diluted with buffer varied from 7.13 to 7.21. The variation in the samples containing antibiotics was small (7.09–7.33), except for oxytetracycline and tetracycline. At the level of 900 μg. per milliliter, the pH was 6.66 and 6.94 for oxytetracycline in trials 1 and 5, and 6.02 and 6.53 for tetracycline in trials 2 and 6, respectively. This reduction may be the result of the inclusion of some citric acid in the antibiotic preparation. The difference in trials is probably because buffer and NaCl were used in trials 1 and 2, while only the buffer was used in trials 4 and 5. Some of the reduction in motility can be accounted for in the reduction in pH to 6.02, but not to the other three pH values; in other studies, a pH of 6.75 was found to be optimum for motility.[9] At levels of oxytetracycline and tetracycline lower than 900 μg. per milliliter, the pH was not lowered appreciably; all values were 7.03 or above, except when 450 μg. of tetracycline per milliliter was used, in which case the pH was 6.83.

The absence of an effect of streptomycin on motility is in agreement with the results of Smith.[7] It also is in general agreement with the results with bull semen,[3,4] in that

TABLE 1—Motility Scores for Semen Stored for 24 Hours at 10 C. Following Dilution with Nine Parts of Buffer with and Without Antibiotics (Values for Duplicate Samples Shown)

Trial (No.)	Antibiotic	Level* None	0.9	9.0	90.0	180.0	450.0	900.0
1†	Oxytetracycline	2+–2+	2+–3–	3––3–	3–3	.....	.....	2–1+
	Oleandomycin	.....	2+–2+	3––3	3+–2	.....	.....	3+–3+
2†	Streptomycin	2+–3–	3––3–	3––3	3––3–	.....	.....	3–3
	Tetracycline	.....	3–3	3–3	3+–3+	.....	.....	1+–1
3†	Chloramphenicol	3–3–	3	2+	3+–3	.....	.....	1+–1+
	Penicillin	.....	3	3–2+	3––3	.....	.....	3––3
	Streptomycin	.....	2–2+	2+–3	3––3+	.....	.....	2––2+
4**	Penicillin	3+–3+	.....	.....	3+–2–	.....	.....	3––3+
	Chloramphenicol	.....	.....	.....	4––3+	3+–3	2+–2	1+–1+
	Oxytetracycline	.....	.....	.....	3–3+	4––3+	2––2–	2––1
5**	Oleandomycin	2–3–	.....	.....	2–3+	.....	.....	3+–3+
	Streptomycin	.....	.....	.....	2+–3–	2–2+	2–2+	2–3–
	Tetracycline	.....	.....	.....	3+–3–	3+–3+	2–2+	2–

* Values are given in micrograms per milliliter for streptomycin, tetracycline, oxytetracycline, and oleandomycin, and in units per milliliter for penicillin and chloramphenicol; † the diluent consisted of equal parts of buffer containing 16.34 Gm. of Na_2HPO_4 and 5.16 Gm. of $NaH_2PO_4 \cdot H_2O$ per liter and of saline solution containing 9.9 Gm. per liter; ** the diluent consisted of buffer without saline solution.

penicillin and streptomycin are not toxic at higher levels, whereas tetracycline, oxytetracycline, and choloramphenicol are. DiLiello *et al.*[3] found oxytetracycline to be also toxic at lower levels (125 to 250 μg./ml.); this toxicity could be the result of low pH, since a strong buffer was not used in the diluent.

Bacterial Count.—The results of eight trials on bacterial counts, when plated on eugon agar, are presented (table 2). In trials 1 and 2, two levels of each antibiotic were tested, except penicillin, for which four levels were tested. Chloramphenicol was tested in trials 3 and 4. Since bacterial counts were not brought to a low level with single antibiotics, combinations were tested. Under trials 3 and 4, every possible two-way combination was used except for oxytetracycline. Levels used were 90 μg., or units, per milliliter, except for penicillin, where 450 units per milliliter was used.

TABLE 2—Bacterial Count, on Eugon Agar, of Semen Stored for 48 Hours at 10 C. Following Dilution with Nine Parts of Buffer with and Without Antibiotics

Antibiotics	Level†	Trial (No.)							
		1	2	3	4	5	6	7	8
Buffer (fresh)		2,000*	2,500	3,300	4,200	1,850	1,340	890	1,520**
Buffer (stored)		3,200	4,400	3,500	2,060	2,580	1,010	500	1,260**
		3,400	.....	4,800	1,960	.....	.....	.....	.....
Penicillin	90	.....	388	.....	.....	.....	.....	.....	.....
	180	.....	0–	.....	.....	900	400	.....	.....
	450	.....	250	390	820	850	300	.....	.....
	900	.....	200	.....	.....	640	77	.....	.....
Streptomycin	90	350	.....	840	610	970	122	.....	.....
	180	810	.....	.....	.....	.....	.....	.....	.....
	450	.....	.....	.....	.....	340	86	.....	.....
	900	.....	.....	.....	.....	48	49	61	26
	1800	.....	.....	.....	.....	.....	.....	.....	13
Dihydrostreptomycin	900	.....	.....	.....	.....	.....	.....	65	37
	1800	.....	.....	.....	.....	.....	.....	34	16
Tetracycline	45	.....	.....	.....	.....	1,700	830	.....	.....
	90	1,010	.....	710	1,160	1,840	860	.....	.....
	180	640	.....	.....	.....	.....	.....	.....	.....
Oxytetracycline	90	.....	491	.....	.....	.....	.....	.....	.....
	180	.....	316	.....	.....	.....	.....	.....	.....
Oleandomycin	90	1,270	.....	1,250	1,170	.....	.....	.....	.....
	180	1,360	.....	.....	.....	.....	.....	.....	.....
	900	.....	.....	870	1,340	2,020	850	.....	.....
	1800	.....	.....	.....	.....	1,950	730	.....	.....
	4500	.....	.....	.....	.....	1,200	310	.....	.....
Chloramphenicol	90	.....	.....	1,300	1,300	.....	.....	.....	.....
	180	.....	.....	1,950	1,570	.....	.....	.....	.....
Penicillin + streptomycin	180 + 90	.....	.....	.....	.....	35	24	.....	.....
	450 + 90	.....	.....	17	85	33	70	.....	.....
	450 + 450	.....	.....	.....	.....	27	14	49	57
	900 + 900	.....	.....	.....	.....	.....	.....	53	10
Penicillin + dihydrostreptomycin	450 + 450	.....	.....	.....	.....	.....	.....	60	18
	900 + 900	.....	.....	.....	.....	.....	.....	45	46
Penicillin + tetracycline	450 + 90	.....	.....	440	1,140	.....	.....	.....	.....
Penicillin + oleandomycin	450 + 90	.....	.....	450	1,410	.....	.....	.....	.....
Penicillin + chloramphenicol	450 + 90	.....	.....	550	1,100	.....	.....	.....	.....
Tetracycline + streptomycin	45 + 90	.....	.....	.....	.....	890	600	.....	.....
	90 + 90	.....	.....	280	400	820	270	.....	.....
	90 + 450	.....	.....	.....	.....	340	49	89	66
	90 + 900	.....	.....	.....	.....	.....	.....	78	37
Oxytetracycline + streptomycin	90 + 450	.....	.....	.....	.....	.....	.....	60	104
	90 + 900	.....	.....	.....	.....	.....	.....	45	58
Oleandomycin + streptomycin	90 + 90	.....	.....	770	1,270	.....	.....	.....	.....
Chloramphenicol + streptomycin	90 + 90	.....	.....	750	1,320	.....	.....	.....	.....
Tetracycline + dihydrostreptomycin	90 + 450	.....	.....	.....	.....	.....	.....	152	141
	90 + 900	.....	.....	.....	.....	.....	.....	90	59
Oxytetracycline + dihydrostreptomycin	90 + 450	.....	.....	.....	.....	.....	.....	154	116
	90 + 900	.....	.....	.....	.....	.....	.....	73	63
Tetracycline + oleandomycin	90 + 90	.....	.....	1,000	1,730	.....	.....	.....	.....
Tetracycline + chloramphenicol	90 + 90	.....	.....	1,000	1,430	.....	.....	.....	.....
Oleandomycin + chloramphenicol	90 + 90	.....	.....	1,630	2,000	.....	.....	.....	.....

* Counts are in thousands per milliliter of semen; † values are given in micrograms per milliliter for streptomycin, dihydrostreptomycin, tetracycline, oxytetracycline, and oleandomycin, and in units per milliliter for penicillin and chloramphenicol; ** average for buffer (fresh) = 2,200, and for buffer (stored) = 2,401.

The only combinations which were better than single antibiotics were penicillin and streptomycin and tetracycline and streptomycin. In trial 3, both oleandomycin and chloramphenicol were relatively ineffective, both singly and in combination. Oleandomycin, at levels as high as 4,500 μg. per milliliter (trials 5, 6), was less effective than several single antibiotics and combinations.

In trials 5 and 6, the highest levels of the two best combinations gave the lowest bacterial counts although, in the case of penicillin and streptomycin, the differences were slight. When penicillin and streptomycin were tested singly, the highest levels were most effective in reducing bacterial counts. A combination of penicillin and streptomycin was superior at all levels to streptomycin, alone or combined with tetracycline. In trials 7 and 8, where higher levels of these combinations were used, there appeared to be no difference when dihydrostreptomycin and oxytetracycline were substituted for streptomycin and tetracycline, respectively. The bacterial count was reduced further by use of the higher level of oxytetracycline or tetracycline with dihydrostreptomycin or streptomycin, but not by penicillin with streptomycin or dihydrostreptomycin. In no case was there complete killing of all bacteria. However, at higher levels of the two best combinations, the colonies present were small. These results do not agree with those of Smith,[7] who found no bacterial growth with 45, 90, and 180 μg. of streptomycin per milliliter. However, he used streaks rather than plate counts, and enough antibiotic may have been carried into the streak to prevent growth of any colonies, even if all the bacteria were not killed. Although the bacterial count was not reduced to zero in this experiment, bacterial growth seemed to be effectively controlled.

In the eight trials, there was an average of 2.2 million bacteria per milliliter of fresh semen. This is markedly higher than the average counts of 73,000, 83,000, and 85,000 found in fresh bull semen.[1, 5, 6] It is of interest that there was only a slight increase in the number of bacteria after incubation for 48 hours at 10 C. There was, however, a qualitative change during incubation. In trials 1 and 2, plate counts also were made on eosin methylene blue agar for coliform-type bacteria. Although the count on this agar of fresh semen was low, it had increased considerably after 48 hours' storage. All the antibiotics tested were effective in controlling growth of this type of organism.

Results similar to but more variable than those shown (table 1), were obtained when thioglycollate medium was used in place of eugon agar. The best results were obtained with the levels and combinations used in trials 7 and 8.

Fertility.—The effect on fertility of using the two best antibiotic combinations is shown (table 3). In all comparisons, the combination of oxytetracycline and dihydrostreptomycin was superior. On the basis of this evidence, it is clear that the addition of this combination of antibiotics results in a marked improvement in fertility from stored semen. The absence of any improvement with penicillin and dihydrostreptomycin is difficult to explain, since this combination has proved beneficial with bull semen and was as effective as oxytetracycline and penicillin in reducing bacterial number.

TABLE 3—Fertility of Semen Stored for Various Times at 10 C. Following Dilution with and Without Antibiotics

Items	1A*	1B†	2**	3**	4A**	4B**
Hours stored	26.5	26.5	20.0	21.0	21.5	21.5
DILUENTS	PERCENTAGE OF FERTILITY‡					
Buffer	4.2(48)	4.8(42)	54.0(50)	55.6(45)	52.0(50)	40.0(55)
Buffer, 450 units penicillin + 450 μg. dihydrostreptomycin/ml.	2.2(45)	2.2(45)	56.0(50)	39.4(33)	23.8(42)	25.0(48)
Buffer, 90μg. oxytetracycline + 900 μg. dihydrostreptomycin/ml.	13.0(46)	18.8(48)	68.0(50)	81.3(32)	66.7(48)	80.4(46)
Unstored, undiluted control	70.0(50)	70.0(50)	97.9(47)	87.0(46)	95.3(43)	95.3(43)

* Diluted 1:10, held under anaerobic conditions; † diluted 1:10, held under aerobic conditions; ** diluted 1:10, held under aerobic conditions, centrifuged after storage at 1200 R.C.F. for ten minutes, brought to original volume with buffer; ‡ first week after insemination. The figure in parentheses indicates the number of eggs laid.

These data do not preclude the possibility that the beneficial effect from oxytetracycline and dihydrostreptomycin is from some mechanism other than antibacterial action. Another possible mechanism is chelation of metallic cations, since the tetracyclines are known to possess chelating properties.[8] Chelation could either remove cofactors such as Mg++ and slow down the rate of metabolism, or remove toxic cations from the solution.

Hatchability of eggs in trials 2, 3, and 4 was measured, and showed little difference between the treatments. Average hatchability of all fertile eggs from unstored semen was 91.0 per cent as compared with 90.8 per cent for fertile eggs from three treatments of stored semen.

SUMMARY

The effect of different levels of seven antibiotics on the motility of chicken spermatozoa, bacterial count, and fertility was studied.

Chloramphenicol, tetracycline, and oxytetracycline reduced motility at 450 and 900 units, or micrograms, per milliliter, whereas penicillin, streptomycin, and oleandomycin did not reduce motility at levels as high as 900 units, or micrograms, per milliliter.

Results of plating semen samples, which had been treated with single antibiotics and all possible two-way combinations, on eugon agar showed the greatest reduction in bacterial count when 450 units of penicillin was combined with 450 μg. of streptomycin or dihydrostreptomycin per milliliter, or when 90 μg. of tetracycline or oxytetracycline was combined with 90 μg. of streptomycin or dihydrostreptomycin per milliliter.

Similar, but less precise, results were obtained with thioglycollate broth.

The bacterial count of fresh chicken semen was found to be much higher than that reported for fresh bull semen.

There was a marked improvement in fertility of semen diluted with a buffer containing oxytetracycline and dihydrostreptomycin as compared with buffer without antibiotics and buffer with penicillin and dihydrostreptomycin.

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The Isolation and Identification of Ornithosis Infection in Turkeys by Tissue Culture and Immunocytochemical Staining

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LABORATORY CONFIRMATION of ornithosis infection in turkeys currently depends on the isolation of the agent in eggs or mice, the detection of serum antibodies by indirect [2] or direct [1] complement-fixation or agglutination [12] tests, and by the use of skin tests. Comparison of the available procedures indicates that reliable and economical methods for detecting ornithosis infection are not yet available.[17]

Coffin [4] has recently emphasized the utility of immunocytochemical staining in veterinary research and medicine. Increasing evidence shows the value of this method in the rapid diagnosis of bacterial and viral infections. *Malleomyces pseudomallei*,[17] *Pasteurella pestis*,[20] *beta* hemolytic streptococci,[10, 15] and enteropathogenic *Escherichia coli*[19] have been visualized in clinical or field specimens by this method. The viruses of influenza,[13] atypical pneumonia,[14] canine distemper,[5] and measles [6] also have been identified by this method in direct and cultured specimens.

Members of the lymphogranuloma-psittacosis group should be particularly suitable for rapid diagnosis by immunocytochemical staining because of the development of typical structures in infected animals or cultured cells. Psittacosis (ornithosis) virus has been propagated in various cell cultures, and the psittacosis (strain 6BC) and feline pneumonitis viruses have been stained with fluorescent antibody by both the indirect and direct methods.[8, 9]

This report describes both the growth in yolk sac explants of ornithosis virus from clinical specimens taken from artificially infected turkeys and the recognition of the developmental forms with the aid of fluorescein-conjugated antiserum. Preliminary studies leading to the procedures described here were carried out with feline pneumonitis under similar conditions.[9]

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MATERIALS AND METHODS

Ten 14-week-old turkeys of the white, medium-weight type were inoculated intratracheally with allantoic fluid containing about 2.5×10^5 embryo lethal doses of egg-propagated ornithosis virus (Jo strain). Cloacal and tracheal swab specimens were collected and stored in rubber-stoppered test tubes on dry ice or at −40 C. until used. Direct smears of the tracheal swabs were also prepared. Specimens were collected at days 0, 3, 7, 10, and 21. Serum samples were collected as indicated (table 1).

Turkey ornithosis immune serums, showing complement-fixation titers in dilutions of about 1:160, were conjugated with fluorescein in the usual manner.[18] The conjugated antiserums were absorbed with acetone-dried chicken liver powder and yolk sac powder to remove nonspecific staining affinities.[19]

Yolk sac explants, cut from the avascular yolk sac membranes of 4- or 5-day-old chicken embryos, were imbedded in plasma on cover slips in Leighton tubes and incubated in a roller drum with chicken serum in Hanks' balanced salt solution (BSS) as described by Weiss and Huang.[16] After 5- to 10-mm. colonies had developed, they were inoculated with material washed out of the swabs with BSS. Bacterial contamination was partially controlled with 50 μg. of dihydrostreptomycin per milliliter of medium. After 48 hours, the explants were fixed in acetone and stored over anhydrous calcium sulfate (Drierite*) in darkness at room temperature until stained. Duplicate tissue culture tubes, each containing a cover slip bearing two or three explants, were routinely inoculated with each specimen. Considerable variation in infection was seen at times among these replicate explants, but observation of as many as three would usually reveal a heavily infected explant, if a marginally infected one was first observed.

Immunocytochemical staining was by the commonly described procedure: The explant was flooded with the fluorescein-conjugated antiserum, reconstituted to its original volume. After approximately one hour in a moist chamber, the explant was washed with three or four changes of buffered saline solution, pH 7.0, over a period of about ten minutes and mounted in buffered glycerol, pH 7.0.

Microscopic observation of the preparations was

* Drierite is produced by the W. A. Hammond Drierite Co., Xenia, Ohio.

by filtered, ultraviolet illumination of a 300 to 400 mμ wave length, obtained with a water-cooled 1,000 W mercury vapor lamp. A cardioid dark-field condenser was used. For photomicrography, 35-mm. color film, with an exposure index of ASA 100, was used. Exposures of two minutes were usual. No color correction filters were employed. The accompanying figures were prepared by conversion of these color transparencies to black and white.

RESULTS

Photomicrographs of fluorescein antibody-stained yolk sac explants (fig. 1–4) show the various appearances of the explants after infection with ornithosis virus by means of cloacal specimens from turkeys inoculated three days previously. Infected cells are recognized by the typical cytoplasmic inclusions and may be surrounded by cells showing no evidence of infection. Typical inclusions may not be stained with the fluorescent antibody. On occasion, large plaque-like bodies, presumably inclusions, remained for the most part unstained (fig. 4). This may be due to an impermeability of the cells to the antibody in spite of the acetone fixation and air drying or a denaturation of the inclusion material.

Controls consisted of explants inoculated with the normal tracheal and cloacal swab specimens. Though the nonspecific inclusions described by Weiss and Huang [18] for this type of preparation were occasionally present in these explants, they could be easily differentiated by their lack of fluorescein staining with conjugated antiserum which had been absorbed with tissue powder. Degeneration of the explant by excessive bacterial contamination from normal or ornithosis-infected specimens in no way resembled the explants infected with ornithosis where the bacterial contamination was controlled, as shown (fig. 1–4).

When the cloacal and tracheal specimens were cultured (table 1), nine of the ten tracheal specimens taken on the seventh day showed evidence of infection with ornithosis virus by the method described, while five of the seven cloacal specimens taken at the same time were positive. Seven of the ten cloacal specimens, collected three days after the turkeys were inoculated, were positive by the fluorescein-antibody technique. Only four tracheal specimens obtained on the third day were examined and all were negative. The cloacal specimens of turkeys 520, 536, and 537, which were negative at three days, were positive on the seventh day. The characteristically heavy infection of explants was not obtained with cloacal specimens from turkey 537 when they were finally examined after about 80 days of storage.

None of the four tracheal specimens taken on the third day yielded an infected explant, although these were examined after 30 days in storage, an interval permitting survival of sufficient infectious material in other specimens to produce heavy infections of the explants.

The fluorescent antibody technique proved unsatisfactory when direct tracheal smears were examined. Intact cells containing the characteristic developmental forms could not be found, although occasional fluorescein-stained structures, possibly representing infected cellular fragments or bacteria, were seen.

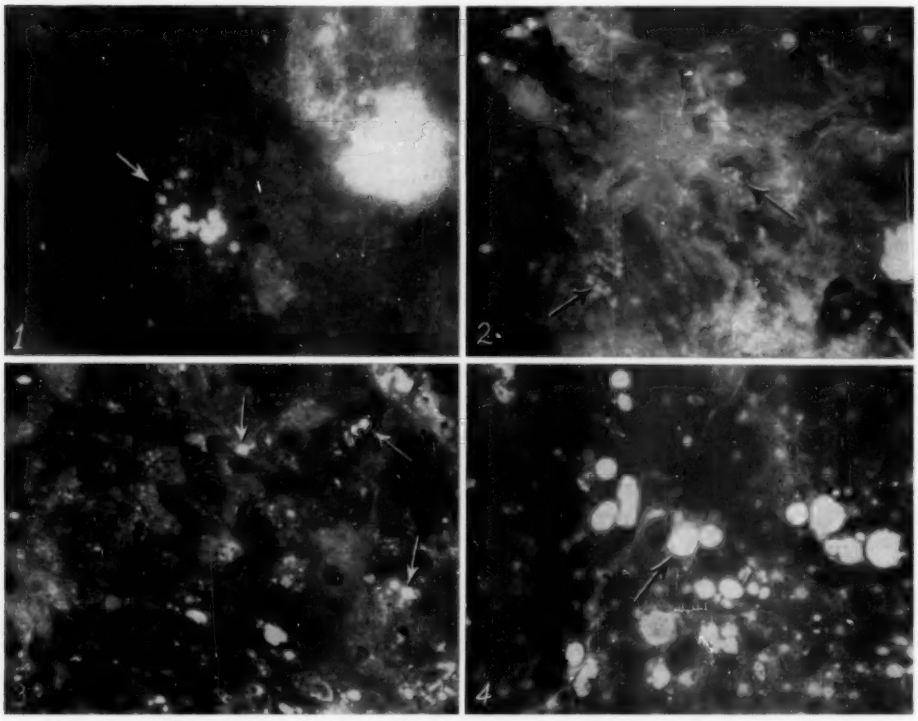
Serological studies were carried out on

TABLE 1—Fluorescein Antibody Staining of Yolk Sac Tissue Cultures and Indirect Complement-Fixation Tests with Specimens from Turkeys Experimentally Infected with Ornithosis Virus

Turkey No.	Specimens obtained following inoculation										
	Day 0			Day 3		Day 7			Day 10		Day 14
	Trachea	Cloaca	Serum	Trachea	Cloaca	Trachea	Cloaca	Serum	Trachea	Serum	Serum
498	—*	—	0†	...	+++	—	—	4	+	32	256
500	—	—	0	—	+++	+	...	8	+	64	128
506	—	—	0	...	+++	+	—	16	+++	8	16
519	—	—	0	...	+++	+	+	16	—	64	128
520	—	—	0	...	—	+	++	4	—	16	16
524	—	—	0	—	+++	+++	...	8	+++	16	8
528	...	—	0	—	+	+++	+++	4	...	8	16
536	—	—	0	...	—	+++	+++	4	...	32	32
537	—	—	0	...	—	+	+	4	—	...	32
543	—	—	0	—	+++	+++	...	0	...	8	16

* All tissue cultures examined after incubation at 37 C. for 48 hours. Approximate number of cells showing fluorescein staining: 10 = +; 10 to 50 = ++; >50 = +++; — = no evidence of infection; ... = not tested.

† Figures indicate reciprocal of highest serum dilution giving positive reaction by the indirect complement-fixation test.



Inclusions of fluorescent antibody-stained ornithosis virus (Jo strain) in cells of yolk sac explants, infected by means of cloacal specimens obtained from turkeys inoculated three days previously. Arrows indicate examples of such inclusions.

Fig. 1—Single cell containing many spherical, yellow-green staining inclusions. x 246.

Fig. 2—Area of an explant with a number of cells containing asymmetrical fluorescein-stained inclusions. x 58.

Fig. 3—Area of an explant inoculated with a specimen from another turkey, resulting in a larger number of infected cells. x 58.

Fig. 4—Area of a duplicate of the explant shown in figure 3. Though the large plaques are probably stages of inclusion bodies, only the indicated structure and the several small inclusions stained yellow-green. x 58.

the 10 turkeys. The indirect complement-fixation test,[2] which is the generally accepted serological test for ornithosis in turkeys, was used. As indicated (table 1), only 2 of the 10 turkeys (506 and 519) had titers as high as 1:16 seven days after inoculation with the virus. In 9 of the 10 birds there was evidence of infection in specimens taken at this time, using the combined tissue culture and Coons technique. An infective cloacal specimen had been obtained previously from the other turkey (498). By the tenth day, 6 of 9 birds tested had indirect complement-fixation titers of 1:16 or greater; by the fourteenth day, all but 1 of the turkeys showed such titers.

In 5 turkeys (498, 500, 524, 528, and 543), diagnosis was established by tissue culture and the Coons technique, using cloacal specimens taken on the third day after inoculation. Antibody titers of 1:16 or greater were not obtained for any of these turkeys until ten or 14 days after inoculation. In the 3 turkeys in which diagnosis by tissue culture and the Coons technique was first established seven days after inoculation, titers of 1:16 and 1:32 were first demonstrated on the tenth day for 2 of these turkeys (520 and 536, respectively). A tenth-day blood sample was not available from the third turkey, but this bird (537) had a titer of 1:32 at 14 days following inoculation of the virus.

The remaining 2 turkeys (506 and 519), which gave evidence of infection on the third day by tissue culture and immunocytochemical staining, showed titers of 1:16 on the seventh day.

DISCUSSION AND SUMMARY

Demonstration of rapid etiological diagnosis of ornithosis in turkeys was accomplished without difficulty by culture of tracheal and cloacal specimens on yolk sac explants, with observation of the infected explants with the aid of fluorescent antibody. The trachea proved to be a suitable source of swab specimens after the occurrence of overt signs. The observation that early cloacal swabs are suitable may be of more practical value. The lack of infectivity of tracheal swab specimens taken at three days is striking, considering that heavy intratracheal inoculation with stock virus was used to infect the turkeys. Cloacal swab specimens taken at the same time were highly infectious. Tracheal swab specimens, taken seven days postinoculation, were positive by the tissue culture-fluorescent antibody technique for 9 of the 10 turkeys. By utilizing both tracheal and cloacal swab specimens, all turkeys were found to be positive by the seventh day.

The early concentration of virus in the cloacal specimens might not occur with an ornithosis virus less virulent than the Jo strain used in these studies. However, none of the turkeys studied died as a result of infection and were still alive four months after initiation of the experiment. A relatively mild infection may have resulted under the conditions of these studies.

Comparison of the tissue culture and immunocytochemical findings with indirect complement-fixation titers indicates that the former may be a more reliable means of diagnosis. Moreover, the specimens for inoculation of the tissue culture explants are useful earlier in the course of the infection and can be obtained by inexperienced personnel.

Higher concentrations of dihydrostreptomycin might be used to control bacterial contamination in the cloacal content, since we have found that 25 mg. per milliliter of this antibiotic in cloacal fluid permits isolation of the virus in developing chicken embryos. Variation in susceptibility of different strains of ornithosis virus to this antibiotic has not been reported.

Though the yolk sac explant technique of Weiss and Huang [18] yields monolayers of large entodermal cells that are well adapted to fluorescent antibody staining and microscopic observation, a cell culture procedure involving trypsinization with the development of monolayers from the resulting cell suspensions might be preferable. Preliminary trials indicate that satisfactory fluorescein-antibody staining of developmental forms of stock Jo and 6BC strains of ornithosis virus can be carried out with Earle's L cells. Whether these cells would permit growth of developmental forms of virus in the presence of extraneous materials found in cloacal content or other clinical specimens will require investigation.

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Experimental Distemper in Mink and Ferrets. I. Pathogenesis

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IN THE STUDY OF DISEASE, the investigators working with natural clinical material are often confronted with limitations of knowledge of such essentials as the exact time of exposure, size of the infecting dose, portal by which the infectious agent entered the host's body, route of migration taken by the invading agent through the host's body, speed of migration, tissues most favorable to growth of the agent, and tissues or organs most seriously damaged by the infection.

Experimental procedures can be devised to recreate the natural disease, while permitting experimental control of various factors, and much fundamental information may be obtained from such experiments.

Fenner's work[2] on ectromelia of mice is a classic example of such a procedure. Investigations into the natural history of poliomyelitis virus have contributed to a truer understanding of its pathogenic activity.[3,6-8] Although the life histories of many metazoan parasites have been worked out in minute detail, comparatively few investigations on the life histories of viruses in the bodies of their hosts have been made.

The experiments described deal with a series of investigations into the life history of distemper virus in two natural hosts—the mink and the highly susceptible ferret.

MATERIALS AND METHODS

All experiments were performed with Green's distemperoid strain of distemper virus, chosen because of its uniformly high degree of virulence for ferrets and mink. The mink and ferrets used were raised in colonies where distemper had never been recognized. Most of the animals were approximately 10 weeks old. On some occasions, older animals were used.

To test for viral activity, to assure a uniform virulence, and to provide ample infectious material for exposure of experimental principals, susceptible ferrets were inoculated subcutaneously with the distemperoid vaccine. These animals showed signs of illness in eight to ten days. At the height of the disease, a few days later, the ferrets were killed. The spleens and lungs were then removed, ground, and diluted to 10^{-1} in tryptone broth containing 2,000 units of penicillin G and 20,000 μg. of dihydrostreptomycin per milliliter. After the larger suspended tissue fragments had settled, the supernatant fluid was placed in a No. 40 DeVilbis nebulizer. The nebulizer was connected to a short-stemmed funnel by a 3-inch length of wide-bored rubber tubing. The bell of the funnel was covered with rubberized fabric in which a 2-inch slit was cut to admit an animal's head. The infective aerosol was thus blown directly into the animal's face for one minute (sometimes longer)

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TABLE 1—Titers of Distemper Virus in Tissues of Ferrets Exposed by Aerosol Inhalation

Postexposure day	Viral titers of tissues					
	Nasal	Lung	Spleen	Liver	Brain	Blood
2	1:5 or >	1:5 or >	1:5 or >	.....	.....	1:5 or >
3	$<10^1$	10^3	10^1 (10^2)	10^2 or >	10^2	10^3
4	10^2	10^4	10^4	10^2 or >	10^2 or >	10^2
5	10^3	10^5	10^3 (10^4)	.....	.....	.....
6	10^4	10^6	10^6	10^3 or >	10^3	10^3
7	10^5	10^5 (10^6)	10^3 (10^4)	.....	.....	.....
8	10^4 (10^5)	10^6	10^4	10^4 or >	10^3	10^3
10	10^4 (10^5)	10^6	10^2 (10^3)	10^5	10^6	10^4
11	10^6	10^6	$<10^5$ (10^3)	.....	.....	.....
12	10^6	10^7	10^6	10^5	10^6	$<10^4$ (?)
13	10^7	10^5 (10^7)	$<10^5$	.....	.....	.....

Titers shown in parentheses represent probably true titers when true titers were not detected at the dilutions assayed.

TABLE 2—Titers of Distemper Virus in Tissues of Mink Exposed by Aerosol Inhalation

Post-exposure day	Viral titers of tissues									
	Nasal	Lung	Kidney and bladder	Blood	Brain	Liver	Muscle	Adrenal gland	Salivary gland	Thyroid gland
2	10^1	10^2	$<10^1$	$<10^1$	$<10^1$	.....	.....	.....	.....	.....
3	.....	.....	.....	10^2	.....	.....	.....	.....	.....	.....
4	10^2	10^3	10^2	10^3	10^1	10^2	$<10^1$	.....	.....	.....
6	10^3	10^4	10^3	10^3	10^3	10^2	$<10^2$ (?)	.....	.....	..
8	10^4	10^5	10^4	10^3	10^2	$<10^3$ (10^2)	10^3	.....	.....	.....
10	10^5	10^6	10^3	10^2 to 10^4	10^3	10^4	10^3	.....	.....	.....
12	10^4	10^7	10^3	10^3 (10^4)	10^4	10^5	10^3	.....	10^4	.....
16	10^6 (10^7)*	10^7	10^5	10^4	10^5	10^5	10^3	10^4*	.....	.....
17	.....	10^2 (?)	.....	$<10^1$ (0) to 10^2	.....	10^2	10^1	.....	.....	$<10^4$*
18	10^4*	.....	.....	.....	..	.....	.....	10^3*	.....	.....
20	$<10^5$ (10^4)	$<10^5$ to 10^6	$<10^3$	$<10^1$ (0)	$<10^3$	.....	.....	.....	.....	.....
23	$<10^5$	$<10^6$	$<10^3$	.....	.....	.....	.....	.....	.....	.....
36	10^5 or >*	.....	.....	.. ..	.....	.....	.....	$<10^2$*	.....	$<10^2$*

* Titers of tissues from mink 6 to 7 months old. All other titers are of tissues from mink 10 to 20 weeks old.

Titers shown in parentheses represent probably true titers when true titers were not detected at the dilutions assayed. Tissues harvested on day 17 or later were assayed individually; those harvested before day 17 were assayed in pools from 2 animals.

at 3 to 6 lb. of pressure per square inch. This procedure was originally described by Sinha *et al.*[9]

The aerosol-exposed mink or ferrets (principals) were placed in battery cages, constructed so that there was no contact between animals although air could circulate from cage to cage. These animals were exsanguinated at intervals by cardiac puncture, with a hypodermic needle. Principals were usually killed beginning on postexposure day 2. Tissues were removed aseptically and prepared for subcutaneous injection into ferrets (assay animals), to determine whether virus was present and the amount of such virus. Tissues were not saved from the aerosol-exposed principals that died, unless such material could be obtained in a fresh state. Tissues assayed for virus content were ordinarily pooled from 2 aerosol-exposed principals, but occasionally from only 1 animal. The ferrets to be used for assay were placed in cages in a spacious open yard, so that accidental infection was minimal. Ferrets that showed signs of illness after postexposure day 15 were considered accidentally infected. Serial tenfold dilutions were used in the assay of viral content of tissues. Occasionally, the end point of viral content was missed. If this happened, a reassay was performed whenever possible.

RESULTS

Distemper virus was detected in every tissue assayed, although it appeared sooner in certain tissues than in others. Virus propagation was more rapid in ferrets than in mink, but the virus attained about the same ultimate titer in both species.

The respiratory system proved to be the most favorable site for the multiplication of distemper virus; a regular, exponential propagation rate was found in lungs of both mink and ferrets, but the titer rose more rapidly in ferrets and attained a titer of 10^6 on postexposure day 6. The maximal titer of 10^7 was attained in the lungs of ferrets on day 12. A titer of 10^6 was not attained until day 10 in mink, but the maximal titer of 10^7 was attained on day 12, as in ferrets.

Virus was detected in blood on postexposure day 2 in ferrets and on day 3 in mink. In other respects, the propagation curve of distemper virus in blood was similar in both species through day 12, and never exceeded 10^4 in either species. In mink living after postexposure day 16, the virus titer of blood fell rapidly. Ferrets were not available for blood samples after day 12.

The titer of distemper virus in nasal tissue also reached a maximum of 10^7,

but the propagation rate was less regular. High titer was attained later than in lung tissue. Livers and brains of both species were moderately favorable sites for propagation of virus. Skeletal muscles, pooled kidneys and bladders, and adrenal, thyroid, and salivary glands (of mink only), and spleens (of ferrets only) were found to be infected in low titers, with a few exceptions (tables 1, 2).

DISCUSSION AND CONCLUSIONS

Investigation of the pathogenesis of distemper in mink and ferrets has produced evidence to support the hypothesis that the virus, while essentially a parasite of the respiratory system, is a pantropic agent capable of invading all parts of the body.

The route of migration is via the blood stream; viremia has been demonstrated in ferrets on postexposure day 2, and in mink on day 3. The viremia persists throughout the course of the disease until eliminated by antibody in the blood, or until death of the host.

Fenner [2] demonstrated a step by step invasion of the body of the mouse by ectromelia virus. After inoculation into the skin, the virus multiplied (1) at the site of inoculation, (2) in the regional lymph node, from which it invaded the blood stream, producing a primary viremia, (3) in the liver and spleen, from which it reinvaded the blood stream several days later, producing a secondary viremia, and (4) in the skin over the entire body infected by the virus during its second invasion of the blood.

In our work, evidence was accrued which showed that, in distemper, the blood stream is invaded soon after the virus has begun to multiply in the respiratory system and the virus persists in the blood constantly thereafter, with small changes in concentration after day 3 or 4.

Liu and Coffin [4] studied the pathogenesis of distemper in ferrets by the fluorescent antibody method. They reported that viremia appeared before the fever and persisted until the death of the animal. They did not state on which day viremia or fever was detected. Although there is some difficulty in obtaining accurate temperatures of ferrets, Belcher's work [1] indicates that an increase in body temperature may be expected about five days after exposure to distemper virus. Assuming that Liu and Coffin detected virus on postexposure day 4, the method employed here would be more effective than the fluorescent antibody method in detecting distemper virus in low concentrations, such as occur in all tissues for several days after exposure.

The earlier detection of distemper virus in respiratory and urinary epithelia of susceptible animals by the method employed in this experiment is further proof that the method is more effective than the fluorescent antibody method, which revealed viral antigen in those tissues one week postexposure. The early appearance of distemper virus in brain, liver, spleen, and kidneys and bladder after its appearance in the blood suggests that no barrier, such as the blood-brain barrier described by Sabin,[5] exists against distemper virus.

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Comparative Histology of the Kidney of Domestic Animals

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A COMPARATIVE STUDY of the microscopic structures of the kidney of the horse, ox, sheep, goat, pig, dog, and cat is reported. The investigation was undertaken because a survey of the literature failed to reveal a thorough study of this subject.

REVIEW OF LITERATURE

Chauveau [5] described the capsule of the horse kidney as a fibrous membrane which intimately unites with the parenchyma of the kidney by numerous prolongations. According to Tereg,[18] the capsule of all mammalian kidneys has two easily separable layers. Tereg [18] and Trautmann and Fiebiger [19] also observed smooth muscle fibers in the deeper portions of the capsule of the ox and sheep.

Langham *et al.*[10] observed that the diameter of the bovine renal corpuscle enlarges from 78 μ at birth to 221 μ at 6 years of age. Rytand,[14] using maceration techniques, found glomeruli that were 150, 180, 166, and 244 μ in diameter in the cat, dog, pig, and ox, respectively. In the cat kidney, the glomeruli in the corticomedullary zone usually are larger than those located more peripherally (Shonyo and Mann [17]).

Bensley and Bensley [2] found a small amount of connective tissue in the intercapillary or axial space of the glomerulus of man and mammals. They observed that a layer of sparse, delicate, reticular fibers surrounds the entrance of the vessels and extends into the lobules of the glomerulus.

Goormaghtigh [8] failed to demonstrate acidophilic or basophilic secretion granules in the juxtaglomerular apparatus of the dog like those he observed in the rabbit.

Modell [13] observed fat in the kidneys of young kittens, but only at the junction of the proximal tubule and Henle's loop. As the cats grew older, however, fine granules of fat seemed to extend toward the glomerulus. Foot and Grafflin [6, 7] observed fat globules in the proximal tubule of the kidney in both cats and dogs. In the cat, the entire pars convoluta and the upper portion of the pars recta of the proximal tubule normally are fat-laden; at the terminal portion, the pars recta is fat-free.

In the dog, the reverse is true; the fat is confined solely to the terminal portion of the pars recta. MacNider [12] made a study of the kidneys from dogs of various age groups. While pups under 4 months of age do not deposit any lipoids in the kidneys, deposition in the epithelium of the proximal convoluted tubules of older dogs increases with age. Lipoidal material also is observed in the loops of Henle. According to Lobban,[11] the anestrous female cat has no fat or only a trace; the estrous female and the normal sexually active male have moderate amounts; the pseudo-pregnant female and surgically castrated male have still more; and the pregnant females and senescent sexually inactive males have the most fat. Lobban associated this phenomenon with the sex hormones, particularly the female hormones.

Langham *et al.*[10] found that the diameter of the straight collecting tubule in the bovine kidney increases from 27 μ at 1 month to 37 μ at 6 years of age. The same investigators observed simple, tall, columnar cells lining the papillary ducts of the bovine kidney.

After Duran-Jorda [4] examined a few hundred routine sections of the kidneys from human beings of all ages and some serial sections from different animals, especially the cat, he was skeptical about the existence of openings of the papillary ducts. More recently, Duran-Jorda's [5] studies have revealed a narrow, elongated orifice from the side of the duct of Bellini into the renal pelvis. The duct of Bellini terminates in a blind cul-de-sac ventral to this opening.

Langham *et al.*[10] observed transitional epithelium covering the surface of the papilla in the bovine kidney.

MATERIALS AND METHODS

The kidneys studied came from 69 animals: 10 horses (5 castrated males and 5 females, all adults), 13 cattle (9 castrated males and 4 females, 16 months to 8 years old), 11 sheep (5 males and 6 females, 3 to 24 months old), 6 goats (4 males and 2 females, 4 to 24 months old), 7 pigs (4 males and 3 females, 3 to 7 months old), 9 dogs (6 males and 3 females, 2 to 36 months old), and 13 cats (2 castrated males, 3 uncastrated males, and 8 females, 7 weeks to 5 years old). These animals were in good nutritional condition and appeared to be free from disease.

In these species, the bovine kidney is the only

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The photomicrographs were made by Dr. Esther M. Smith, Anatomy Department, Michigan State University, East Lansing.

one showing an external lobation. All others have a smooth surface. On longitudinal section, the kidneys of the pig and ox appear multipyramidal. The other species show various modifications, in which the papillae are fused into a single renal crest.

The kidneys were split longitudinally on removal from the freshly killed animals, and fixed in Lavdowsky's mixture for four to six days. Blocks of the fixed kidney tissues were obtained by the technique of Barrie,[2] which insured anatomic continuity of the renal tubules in microscopic sections. The method took into account the gross anatomy of the medulla, so that the tubules and their accompanying vessels could be followed continuously from the papilla to the corticomedullary junction. Small blocks (about 1 cm. wide and 0.5 cm. thick) were cut in the vertical plane of the straight collecting tubules and the papillary ducts in such a way that a portion of the pelvis, the papilla, the medulla, the cortex, and the capsule of the kidney were included. This plane gave a longitudinal section of the uriniferous tubules. In the large animals, these blocks were divided transversely into two halves at the corticomedullary junction to make them smaller for better sectioning. Four blocks were obtained from both kidneys of each animal. Following butyl alcohol dehydration, routine paraffin embedding was used.

Hematoxylin and eosin, Weigert's, Van Gieson's, and Gridley's[37] stains for reticular tissue

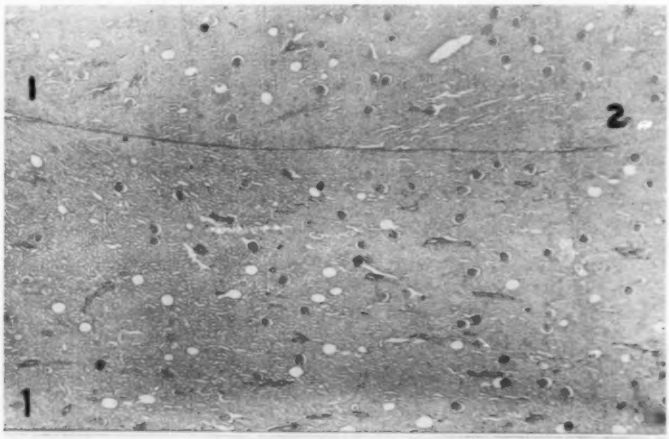


Fig. 1—Connective tissue septum penetrating the cortex of the bovine kidney, showing (1) cleft between lobules and (2) end of collagenous septum. Weigert's and Van Gieson's stains; x 10.

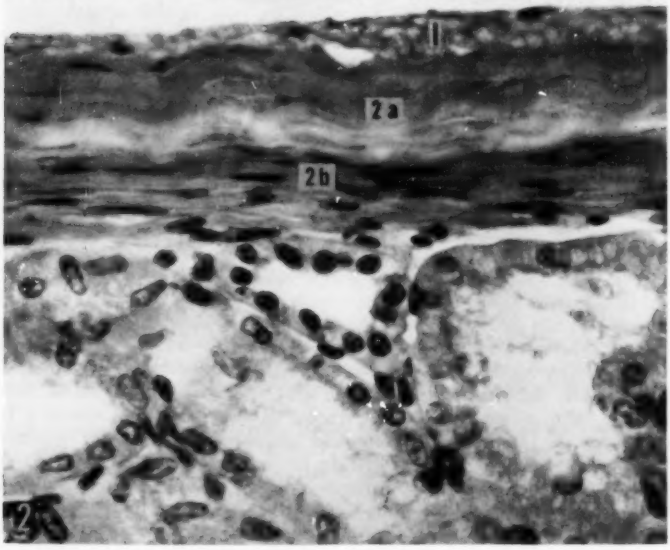


Fig. 2—Capsule of the kidney of a 9-month-old goat, showing the capsula fibrosa (2), its outer (a) and inner (b) layers, and the capsula adiposa (1). Notice the muscle cells in the inner layer of the capsula fibrosa. H & E stain; x 510.

TABLE 1—Diameter of the Uriniferous Tubule, with Epithelial Height *

Species	No.	Renal corpuscle: Cortical d.†	Renal corpuscle: Juxtamedullary d.	Glomerulus: Cortical d.	Glomerulus: Juxtamedullary d.	Proximal convoluted tubule: d.	Proximal convoluted tubule: e.h.**	Thin segment of Henle's loop: d.	Thin segment of Henle's loop: e.h.	Distal convoluted tubule: d.	Distal convoluted tubule: e.h.	Straight collecting tubule: d.	Straight collecting tubule: e.h.	Papillary duct: d.	Papillary duct: e.h.
Horse	10	178	191	159	166	56	15	30	6	50	9	66	14	138	55
Ox	13	181	173	150	141	50	13	26	5	45	8	53	12	91	33
Sheep	11	153	147	123	123	45	11	22	4	36	7	39	10	59	18
Goat	6	158	157	127	126	48	11	24	5	38	8	42	12	57	22
Pig	7	137	156	114	130	45	14	23	5	36	8	50	14	96	21
Dog	9	122	124	98	100	39	11	22	5	33	7	37	9	54	13
Cat	13	96	106	82	87	41	14	31	4	30	7	37	5	60	14

* Values are given in microns, and the average is based on ten counts per animal; † d. = diameter; ** e.h. = epithelial height.

were used routinely. Mast cells were demonstrated with toluidine blue and Löffler's methylene blue solutions.

The diameters of all parts of the uriniferous tubule were measured from random samples seen under the light microscope. The transverse diameters of the longitudinal section of the renal corpuscle and glomerulus were determined at their centers; only those structures which appeared to be cut through the center in a vertical plane were measured. This was designated by the presence of both vascular and urinary poles in the same section of the renal corpuscle.

RESULTS AND DISCUSSION

The Capsule.—The tunica fibrosa of the capsule in all kidneys is thick and composed of two distinct layers. The outer (thicker) layer consists of dense collagenous and a few elastic fibers; the inner (thin) layer is composed of loose collagenous and reticular fibers and a few smooth muscle cells. The reticular fibers from this thin inner layer penetrate the parenchyma of the kidney. At the points of external lobation on the ox kidney, a sheath of collagenous connective tissue extends between the lobules. It decreases in amount and spreads out, losing its identity (fig. 1). The capsula adiposa lies just outside the tunica fibrosa and contains blood vessels and nerves.

The following variations were observed in the layers of the tunica fibrosa: The inner layer is best developed in the horse; in the sheep and goat, both layers are equally developed and well demarcated; in the cat, the inner layer is absent. In the ox, sheep, and goat, the inner layer contains so much smooth muscle that it forms almost a distinct muscular layer in the deeper portion of the capsule (fig. 2). The dog has less smooth muscle fibers than the others except the cat, which has none. While the outer layer of all specimens observed contains some elastic fibers, those of the sheep seem to have the most. Reticular fibers are least developed in the cat.

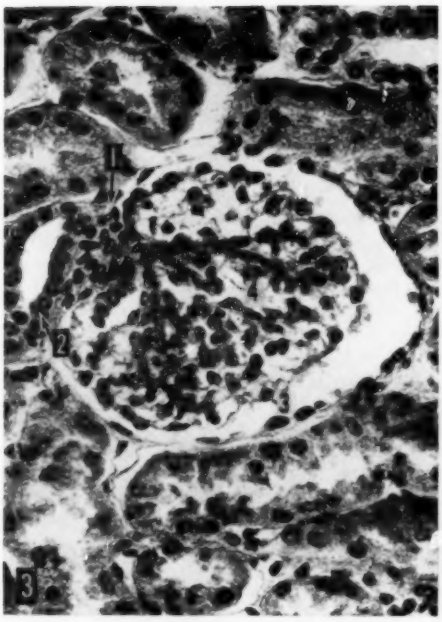
The Renal Corpuscle.—The renal corpuscles of the pig, horse, ox, sheep, and goat are pear shaped; those of the dog and cat are more or less spherical. The average transverse diameters of the cortical and juxtamedullary renal corpuscles of the various animals are shown (table 1). While the juxtamedullary renal corpuscles are larger than the cortical ones in the horse, pig, dog, and cat, the reverse is true in the ox and sheep. The size of the renal corpuscle increases with age, reaching maximum at the adult stage.

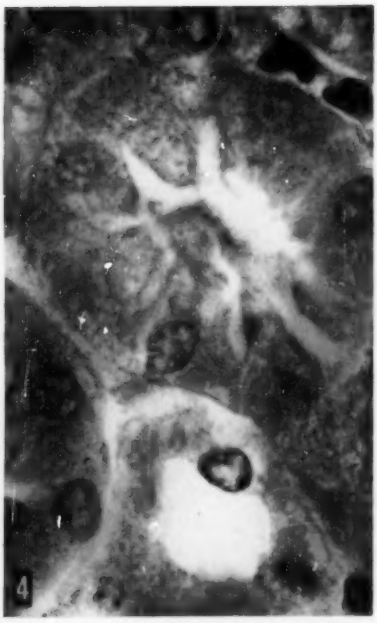
While the glomerulus of the juxtamedullary region in the pig kidney has an average diameter of 130 μ, that of the cortical one averages 114 μ. The juxtamedullary glomeruli are larger than the cortical ones in the pig, horse, dog, and cat, while the cortical glomeruli are larger than the juxtamedullary ones in the ox. In the sheep and goat, no appreciable difference is noticed between the sizes of the juxtamedullary and cortical glomeruli.

The intercapillary or axial space of the glomerulus in all animals contains collagenous and reticular fibers, fibroblasts, and histiocytes.

The three primary membranes of the glomerulus are present in all animals of this investigation. The finer details of these membranes cannot be observed with the ordinary light microscope. Both the parietal and visceral layers of Bowman's capsule in all animals under study have a flattened squamous epithelium. There is more capsular space present in the goat, ox, and sheep than in other animals.

The Juxtaglomerular Apparatus.—The





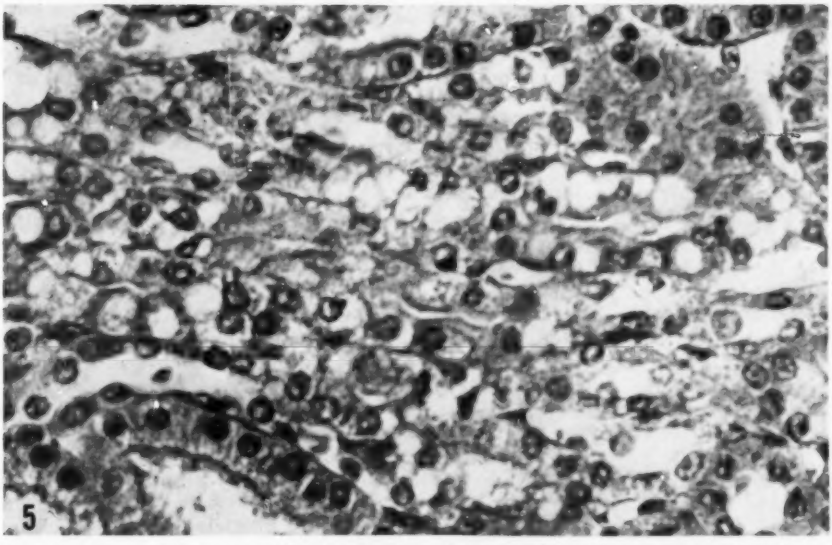


Fig. 3—Kidney of a 5-month-old goat, showing (1) juxtaglomerular apparatus, (2) single-layered macula densa, (3) visceral layer of Bowman's capsule, and (4) endothelial cell. H & E stain; x 350.

Fig. 4—Kidney of a 7-month-old pig. Notice the brush border forming a cluster on each cell of the proximal tubule. H & E stain; x 1,330.

Fig. 5—Kidney of a 4-month-old cat. Notice the fat globules in the pars recta of the proximal tubule. H & E stain; x 500.

nuclei of the juxtaglomerular cells, the modified smooth muscle cells of the media of the afferent arteriole, are spherical and stain deeply with hematoxylin and eosin. In addition to these myoepithelioid cells of the media, some spindle-shaped smooth muscle cells also are observed among them. These cells are closely associated with the macula densa (fig. 3). The only apparent variation is an increase of the number of juxtaglomerular cells in the goat and dog.

The Proximal Convoluted Tubule.—The general structure of the proximal tubule is similar in all species. It is lined by simple truncated pyramidal cells, whose cytoplasm is coarsely granular. These granules are more or less evenly distributed and, hence, basic striations are not distinct. The nuclei usually are basal but, in some cells, they are situated in the middle or even toward the apex of the cells. These nuclei are spherical and stain deeply with hematoxylin. Each nucleus contains a prominent nucleolus and some deeply stained chromatin granules. The lumen contour in the pig is irregular and the brush border forms a separate cluster for each cell (fig. 4). Some of the nuclei of the cells of the proximal tubule are ovoid in the ox and goat. The brush border is uniformly arranged along the apices of the cells of the proximal tubule in all animals except the pig. Basal striations are more distinct in the dog and cat than in others. Fat globules are present in the proximal tubule in the cat and dog (fig. 5).

The Loop of Henle.—Henle's loop is lined by flattened epithelial cells which are markedly less eosinophilic than those of the proximal tubule. The cytoplasm of the cell is more or less clear, with a spherical nucleus which bulges into the lumen, giving it a wavy contour. A brush border is absent and the basal striations are indistinct. The structures of the descending thick limb of Henle's loop are similar to those of the proximal tubule, while the structures of the ascending limb are similar to those of the distal tubule.

The Distal Convoluted Tubule.—The histological structure of the distal tubule is essentially the same for all species. Cuboidal epithelium, 7 μ in height, lines the tubule. In comparison with the proximal convoluted tubule, its lumen is larger and its contour less irregular, the cytoplasm is granular and less eosinophilic, the granules are finer, and the spherical basally placed nuclei are closer to each other. A few ovoid nuclei are present. The nucleus shows one or two prominent nucleoli and densely stained coarse chromatin granules. The cell boundaries are not clear. The brush border is absent, and the basal striations are not distinct.

The Macula Densa.—Except in the horse, the maculae densae of all species are similar. They are closely associated with the juxtaglomerular cells. The cells forming the macula densa are single layered and appear slightly taller than the rest of the cells of the distal tubule. The cytoplasm is faintly stained around the nucleus and contains few fine granules. The nucleus is spherical and contains a densely stained nucleolus and coarse chromatin granules. In the macula densa of the horse kidney, an abrupt change from simple to stratified epithelium takes place. The stratifications are not distinct, as the nuclei are irregularly crowded (fig. 6). The height of the stratified epithelium of the macula densa appears more than double that of the other cells lining the same tubule. The cells of the macula densa have few granules and do not take the stain, especially toward the base. Occasionally, two maculae densae are observed occurring at the same afferent arteriole of the glomerulus in the horse and ox (fig. 7).

The Arched Collecting Tubule.—Except for the diameter and cell height, the structures of the arched collecting tubules of all species are much alike. The diameter is greater and the lumen more regular and wider than that of the distal tubule. It is lined by a simple cuboidal epithelium with distinct cell boundaries. The cytoplasm of the cells lining the arched tubule is faintly stained and devoid of any characteristic granules. The nuclei are either spherical or oval, and are basally placed. Each nucleus contains a nucleolus and some deeply stained chromatin granules. The diameter is greater in the horse and ox than in the pig, sheep, goat, dog, and cat. The cell height is greater in the horse than in the rest of the animals.

The Straight Collecting Tubule.—The lumen of the straight collecting tubule has a regular contour and is lined by a simple cuboidal epithelium which becomes somewhat wider and taller towards the papillary duct. The cytoplasm is clear, faintly

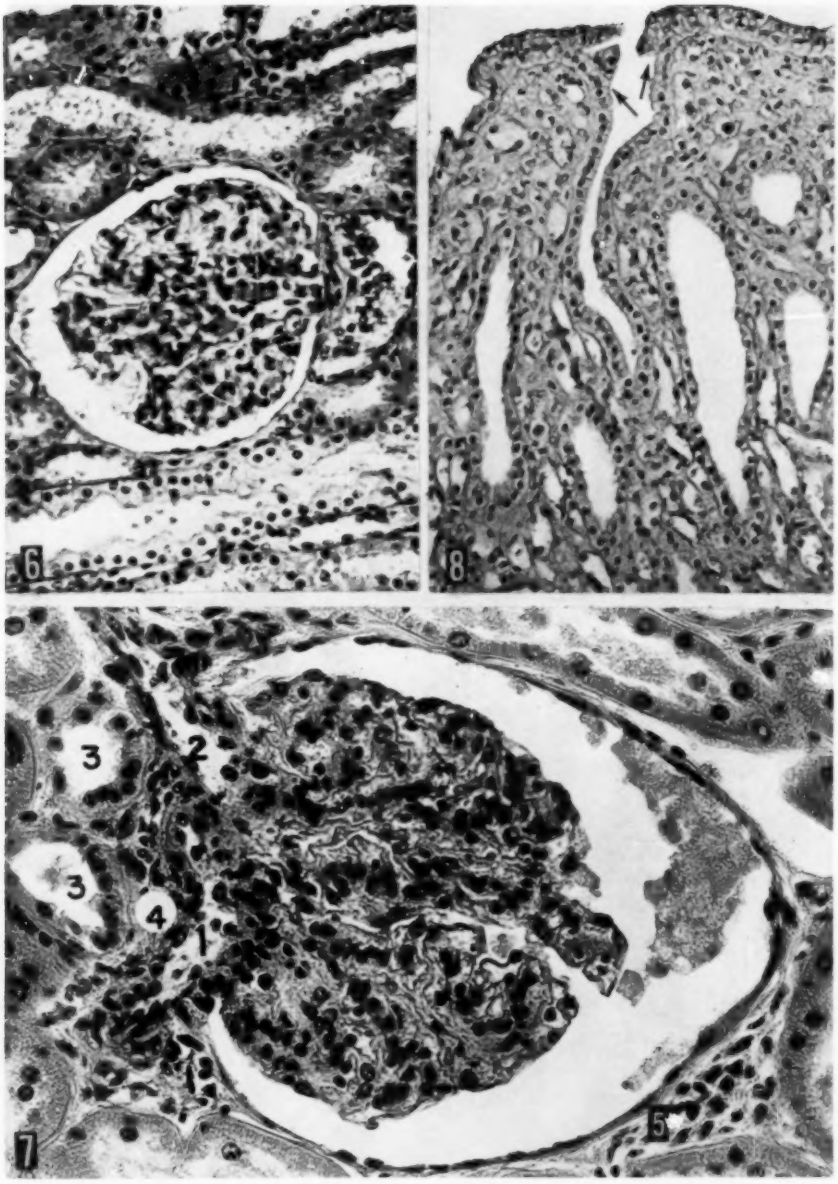


Fig. 6—Kidney of an adult horse. Notice the stratified macula densa. H & E stain; x 200.

Fig. 7—Glomerulus from the kidney of a 6-year-old cow, showing (1) afferent arteriole, (2) efferent arteriole, (3) unusual double maculae densae, (4) cell unit of Goormaghtigh, and (5) Becher's cells. H & E stain; x 280.

Fig. 8—Papillary ducts in the kidney of a 1-year-old dog, showing the junction of simple columnar and transitional epithelium near the pelvis. Notice the beaklike epithelial processes (arrows) at the opening of the papillary duct. H & E stain; x 160.

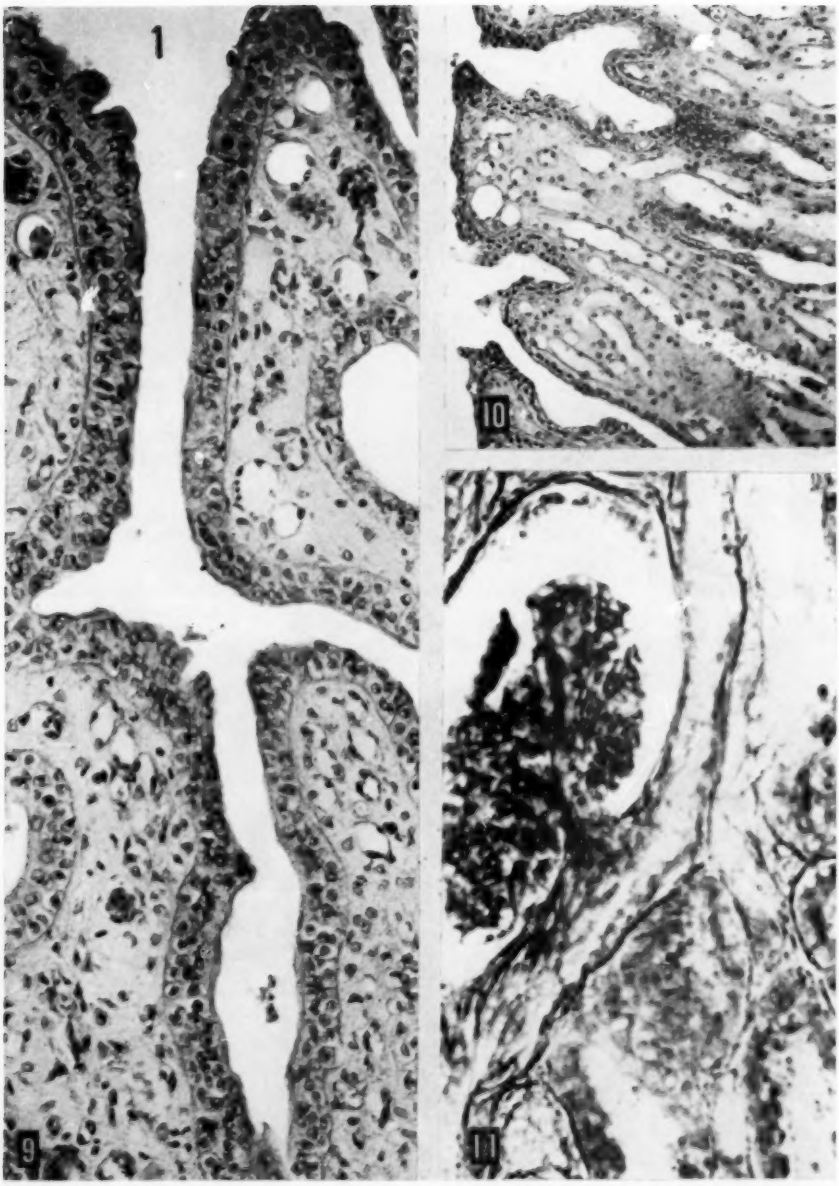


Fig. 9—The papillary ducts in the kidney of a 19-month-old ox, showing the transitional epithelial lining and the opening on the papilla at (1). H & E stain; x 250.

Fig. 10—The papillary ducts in the kidney of a 7-week-old cat. H & E stain; x 110.

Fig. 11—Kidney of a 7-month-old pig, stained for reticular connective tissue. Notice the distribution of reticular fibers in the interstitial spaces of the parenchyma and the glomerulus. Gridley's modification of the silver stain; x 300.

stained, and free from granules. The nucleus contains a deeply stained nucleolus and a few coarse chromatin granules. The cell boundaries are distinct.

The diameters of the straight collecting tubules vary from 66 to 77 μ in the following order: horse, ox, pig, goat, sheep, dog, and cat. The epithelial heights vary from 14 to 5 μ in this order: horse and pig, 14 μ; ox and goat, 12 μ; sheep, 10 μ; dog, 9 μ; and cat, 5 μ. Due to the low epithelial lining, the lumina of the collecting tubules in the cat are larger than those of the sheep, goat, pig, or dog.

The cell boundaries are less distinct in the sheep and cat than in other species. No fat droplets are present in the straight collecting tubules of any of the animals under investigation, as described by Trautmann and Fiebiger.[19]

The Papillary Duct.—The general structure of the papillary duct resembles that of the straight collecting tubule. The diameter and epithelial height are greater than those of the straight collecting tubule. The papillary duct is lined by a simple tall columnar epithelium which becomes transitional near the opening of the duct onto the papilla. The transitional epithelium of the papillary duct continues as the epithelium of the papilla. The epithelium in the papillary duct changes abruptly from a simple to transitional type. The tall columnar cells of the papillary duct are faintly stained and nongranular. The nuclei are large, spherical, and situated almost in the center of the cell. Each nucleus contains a dark-staining nucleolus and chromatin granules. The cell boundaries are distinct and the contour of the lumen is regular. The following species differences occur: The diameter of the papillary duct is largest in the horse (138 μ) and smallest in the dog (54 μ). The epithelium is by far the tallest in the horse and lowest in the dog (table 1). In the horse, ox, goat, and sheep, the papillary duct is lined by a transitional epithelium for a greater distance from its opening on the papilla than that of the pig and cat. The papillary duct of the dog kidney is lined by a simple columnar epithelium throughout its course. At the junction of the simple columnar epithelium of the papillary duct and the transitional epithelium of the papillae in the dog, a beaklike process of the transitional epithelium is observed in longitudinal section (fig. 8).

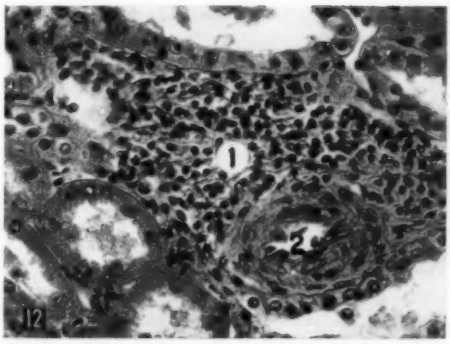


Fig. 12—Kidney of a 6-year-old ox, showing (1) intertubular cell group, or Becher's cells, and (2) interlobular artery. H & E stain; x 259.

Longham *et al.*[10] reported that only tall columnar cells line the papillary duct of the bovine kidney. This study revealed that transitional epithelium may line the papillary duct of the bovine kidney for a distance of as much as 1,500 μ from the opening of the papillary duct (fig. 9). The openings of the papillary ducts in the cat are shown (fig. 10).

The Basement Membrane of the Uriniferous Tubule.—The kidneys of all the domestic animals contain a basement membrane in the uriniferous tubules, although details of its finer structures could not be studied with the ordinary light microscope.

The Papilla.—In the pig kidney, transitional epithelium of the calyx minor reflects over the sides of the papilla. In the angle of reflection, the high transitional epithelium of the calyx minor becomes low and continues as such for some distance along the sides of the papilla. After a short distance, this low transitional epithelium becomes higher and covers the sides and apex of the papilla. The papillary transitional epithelium maintains almost uniform height over the entire surface of the papilla, except on the sides near the angle of reflection. The height of the transitional epithelium of the papilla never acquires the original height of that which lines the calyx minor. The papillae of the other animals are similar to those of the pig, except that the transitional epithelium is higher in the horse and lower in the sheep, goat, dog, and cat.

The Interstitial Connective Tissue.—The

interstitial space of the kidney of all animals contains reticular fibers which extend from the capsule to the apex of the papilla (fig. 11). These reticular fibers are more numerous near the blood vessels and in the interstitial spaces of the medulla. Collagenous fibers surround the large blood vessels, especially at the corticomedullary junction. A few fine collagenous fibers invest the uriniferous tubules and Bowman's capsule. Fibroblasts and histiocytes are also observed in the interstitial spaces. Only the interstitial connective tissue of the dog and cat lack mast cells.

The Intertubular Cell Groups, or Becher's Cells.—According to Schloss,[15] Becher described intertubular cell groups in 1936. These are present in the interstitial spaces of the cortical region between the uriniferous tubules, close to Bowman's capsule, and around the cortical arteries in all species except the cat (fig. 12). The groups surrounding the arteries usually are larger; some of the small groups are encapsulated. The cells have no regular shape, most of them being polygonal, although a few are ovoid. The cytoplasm is faintly eosinophilic and nongranular.

The nucleus is smaller and darker than that of the surrounding tubules. The nuclei of these cells are generally spherical, although a few are ovoid. They are located either in the center or toward one edge of the cell. The nucleus has a characteristic appearance; four or five crescent-shaped, dark nuclei are present along the periphery of the nucleus close to each other and the nuclear membrane. A large, deeply stained nucleolus is situated at the center of the nucleus. The spaces between these nucleoli are unstained and free from chromatin granules. These cell groups are more numerous and better developed in the ox and horse than in the pig. In the ox, the cells are more eosinophilic and contain more cytoplasm than those of the

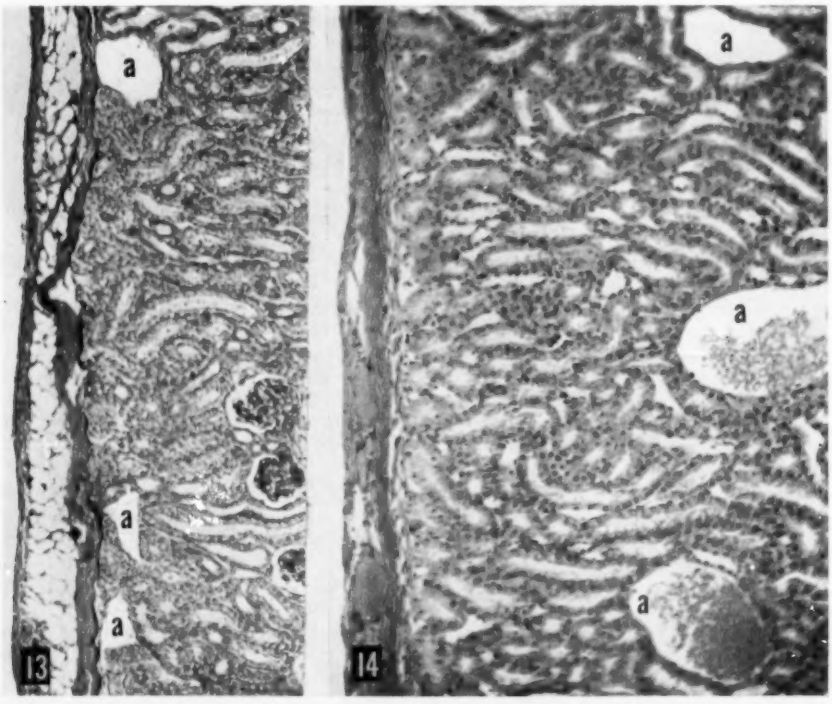


Fig. 13—Cortex of the kidney from a 2-month-old cat, showing the stellate veins (a) in close proximity to the capsule. H & E stain; x 115.

Fig. 14—Cortex of the kidney from a 1-year-old dog, showing the stellate veins (a) some distance in from the capsule. H & E stain; x 115.

pig. In the sheep, goat, and dog, the cell groups are less developed.

Cell Unit of Goormaghtigh.—The cell unit of Goormaghtigh, which consists of a cluster of small cells situated in the triangular space formed by the afferent and efferent arterioles and the macula densa, is present in all seven species (fig. 7). These cells are closely associated with the juxtaglomerular cells and the cells of the macula densa. Becher [16] regarded this cluster of small cells in the human kidney as an area containing nerve receptors.

Sex Differences.—This investigation revealed no sex differences in the structure of the kidney.

Stellate Veins.—The stellate veins show large vascular spaces in the dog and cat. Those in the cat are just under or in the edge of the capsule, while those of the dog appear a short distance in from the periphery (fig. 13, 14).

SUMMARY

Kidneys from 10 horses, 13 cattle, 11 sheep, 6 goats, 7 pigs, 9 dogs, and 13 cats were used in this study. No sex differences were apparent.

Except in the cat, the capsule contains an outer dense fibrous layer and an inner looser layer containing varying amounts of smooth muscle, depending on the species. In the cat, there is only one layer which lacks muscle. The capsule in the ruminants contains the most smooth muscle.

Measurements include the diameter of the glomeruli and all parts of the uriniferous tubule, and the epithelial height. The renal corpuscles vary in size according to the species size, being largest in the horse and cow and smallest in the cat.

The renal corpuscles are largest next to the medulla in the horse, pig, dog, and cat, but those nearest the capsule are largest in the ox.

The juxtaglomerular apparatus is present in all species, including a 2-month-old kitten, a 3-month-old pig, and a 3-month-old lamb.

The diameter of all parts of the uriniferous tubule is greatest in the horse and least in the cat. The epithelial height follows a similar pattern except in the proximal convoluted tubule, in which the epithelium is lowest (11 μ) in the sheep, goat, and dog. Fat globules are present in the proximal convoluted tubule in the carnivores.

The maculae densae are similar in all animals except the horse, which shows nuclei crowded together in such a way as to appear stratified.

Transitional epithelium lines the papillary ducts in all species except the dog, which has only simple columnar epithelium. A valvelike epithelial flap marks the entrance of the papillary duct into the pelvis in the dog and cat.

Intertubular or Becher's cells are present in all the domestic animals except the cat, and are best developed in the horse and ox. Goormaghtigh's cells are present in all species.

The stellate veins are particularly prominent in the dog and cat. In the cat, they are just beneath or in the edge of the capsule while, in the dog, they are located in the cortex a short distance from the periphery.

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Ringworm Fungi of Feral Rodents in Southwestern Georgia

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THE OCCURRENCE, abundance, and distribution of ringworm fungi in feral animals in southwestern Georgia were studied as a cooperative project by the Newton Field Station of the Technology Branch, Communicable Disease Center, and the Microbiology Section of the Communicable Disease Center's Laboratory Branch.

Ringworm fungi pathogenic to man have been isolated from domestic and wild animals by many investigators (Errington,[7] Ainsworth and Austwick,[1] and Emmons[6]). The significance of such findings was shown by Georg,[4] who estimated that 70 to 80 per cent of infections of man in rural areas resulted from contact with infected animals or with materials contaminated by these animals. The ecology of ringworm fungi in commensal rats of this area (Smith *et al.*[11]) and a summary of the isolations from all animals collected in the area through September, 1955 (Menges *et al.*[10]), have been reported.

MATERIALS AND METHODS

All specimens were taken in traps* set 20 feet apart in lines of 25 and maintained for three or four nights. A total of 255 lines was run in 11 types of habitat, representing either stages in plant succession or habitats established by man. Rolled oats and peanut butter were used for bait. All traps were checked each morning, the animals removed, and the traps rebaited. The animals were brought to the laboratory in individual cloth bags and then killed. Using sterile forceps, hairs were plucked from the nose, forehead, middle of the back, and between the forelegs from each animal and sealed in envelopes. At this time, the animal was examined for the presence of skin lesions.

The hair specimens were studied at the Communicable Disease Center Mycology Unit laboratory for the presence of ringworm fungi by direct microscopic examination for fungus elements after treatment with 10 per cent potassium hydroxide, by Wood's lamp examination for fluorescence, and by culturing as described by Georg.[4] The specimens were collected between Oct. 13, 1954, and Sept. 30, 1956. A total of 189 soil samples was collected in 24 areas and cultured at the same laboratory by baiting the soil with hair filaments to determine the presence of keratinophilic fungi (Ajello[2]).

RESULTS

Hair specimens from 996 feral rodents of eight species were examined (table 1). Dermatophytes, or dermatophyte-like fungi, were obtained from 114 (11.4%) of the specimens. However, the direct microscopic and Wood's lamp examinations were negative. The fungi obtained were: 17 isolates of *Trichophyton mentagrophytes*, 14 of *Microsporum gypseum*, and 83 isolates of a red Microsporum, here designated as Microsporum (red variety), considered a new species by Ajello.[3] This fungus is not known to be pathogenic. Recovery of these fungi from four species of rodents, of which 50 or more were tested, ranged from 1.2 per cent for *Mus musculus*

TABLE 1—Results of Survey for Ringworm Fungi in Mammals from Southwestern Georgia

Species	No. examined	Positive (No.)	Positive (%)	*Trichophyton mentagrophytes*	*Microsporum gypseum*	Microsporum (red variety)
Mus musculus	243	3	1.2	1	2	....
Peromyscus polionotus	311	29	9.3	13	6	10
Peromyscus gossypinus	58	20	34.5	2	2	16
Peromyscus nuttalli	1	0	0.0	....	....	....
Reithrodontomys humulis	24	3	12.5	....	2	1
Sigmodon hispidus	351	57	16.2	1	2	54
Neotoma floridana	7	2	28.6	....	....	2
Pitymys pinetorum	1	0	0.0	....	....	....
Totals	996	114	11.4	17	14	83

From the Communicable Disease Center, Bureau of State Services, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Ga., and the Emory University Field Station, Newton, Ga.

The authors acknowledge the assistance of Dr. L. K. Georg and Mr. G. W. Gorman in these studies.

* Young's live traps, No. 15-A, manufactured by Young's Animal Trap Co., Sunnyvale, Calif.

TABLE 2—Number of Ringworm-Infected Mammals Collected from Various Habitats in Southwestern Georgia

Species	Barn-yard	Pasture or meadow	Marsh around pond	Grain or peanut field	Tall weeds	Broom sedge	Broom sedge pine	Pine	Pine hard-wood	Upland hard-wood	Bottom land hard-wood	Totals
Mus musculus	4	5	....	38(1)*	152(1)	35	3(1)	5	..	..	....	243(3)
Peromyscus polionotus	2	10	....	4(1)	227(21)	31(1)	18(1)	4(4)	8	7(1)	....	311(29)
Peromyscus gossypinus	..	....	....	....	4	1(1)	18(9)	3(3)	..	..	32(9)	58(20)
Peromyscus nuttalli	..	....	....	....	....	....	....	.	..	..	1	1
Reithrodontomys humulus	..	6	....	....	2	....	11(2)	5(1)	..	..	....	24(3)
Neotoma floridana	..	....	....	....	....	....	....	..	..	..	7(2)	7(2)
Sigmodon hispidus	1	4	7(1)	....	8(3)	328(51)	3(2)	.	..	..	....	351(57)
Pitymys pinetorum	..	....	....	....	....	....	1	..	..	..	....	1
Totals	7	25	7(1)	42(2)	393(25)	395(53)	54(15)	17(8)	8	8(1)	40(11)	996(114)

* No. of infected animals indicated in parentheses.

to 34.5 per cent for *Peromyscus gossypinus*; for four other species, the number of specimens examined was too small to give significant prevalence rates.

The ecological distribution of the specimens collected and of the host animals is shown (table 2). A total of 859 (86%) of the 996 animals collected and 99 (87%) of the host animals were collected from tall weeds, broom sedge, broom sedge pine, and pine woodlands. These habitats represent progressive stages in the plant succession of abandoned fields toward climax forests. The percentage of positive animals from the various habitats increased sharply with the progression of the plant succession toward pure pine, but the total number of animals caught decreased rapidly (table 2). The prevalence of dermatophytes and the Microsporum (red variety) and the number of specimens examined from four of the major stages of plant succession were as follows: 6.4 per cent of 393 from tall weeds, 13.0 per cent of 395 from broom sedge, 28.0 per cent of 54 from broom sedge pine, and 47.0 per cent of 17 from pine. Of the 40 animals from bottom land hardwoods, 28 per cent were positive. Only 4 of the 97 animals (4.1%) from other habitats were positive.

Of the 114 isolates, 83 were Microsporum (red variety). This fungus was obtained from 1 (4.1%) of 24 *Reithrodontomys humulis*, 10 (3.2%) of 311 *Peromyscus polionotus*, 16 (27.5%) of 58 *P. gossypinus*, 54 (15.2%) of 351 *Sigmodon hispidus*, and 2 (28.5%) of 7 *Neotoma floridana*. Seventeen isolates were *T. mentagrophytes*; these were found on 13 (4.2%) of 311 *P. polionotus*, 2 (3.4%) of 58 *P. gossypinus*, 1 (0.3%) of 351 *S. hispidus*, and 1 (0.4%) of 243 *M. musculus*. Fourteen isolates of *M. gypseum* were obtained from 2 (8.3%) of 24 *R. humulis*, 6 (1.9%) of 311 *P. polionotus*, 2 (3.4%) of 58 *P. gossypinus*, 2 (0.6%) of 351 *S. hispidus*, and 2 (0.8%) of 243 *M. musculus*. Skin lesions were observed on none of the animals.

Of the 189 soil samples tested, 100 were collected in pine woodlands; the remaining 89 were gathered around rodent burrows in fields and bottom land hardwood forests. *Microsporum gypseum* was isolated from 17 of the soil samples collected in fields and bottom lands. All the pine woodland specimens failed to yield keratinophilic fungi.

Discussion

In view of the absence of any signs of infection on the animals and the failure to detect any evidence of the organisms by microscopic examination of the hairs, it seems possible that the animals examined during this investigation may not have been infected but served only as carriers of spores or other fungus elements picked up from the environment. Ajello[2,3] presented evidence that *M. gypseum* basically is a saprophytic fungus and postulated that soil is the main source of infections by this fungus in both man and animals. The Microsporum (red variety) has been isolated from soil (Cooke[5]), but attempts to infect animals with this fungus have not been successful and no cases in man have been reported. If the wild mammals considered here were not infected with ringworm fungi but only carried fungus elements in their fur, then their main role in propagation of the disease would be in dissemination of these elements picked up from the environment.

Trichophyton mentagrophytes is an occasional agent of ringworm in cattle (Georg[8]) and horses (Georg *et al.*[9]). These animals most frequently graze or work in pastures or areas with a cover of tall weeds. *Peromyscus polionotus* and *M. musculus* are the most abundant rodents in such areas. *Mus musculus* is a sleek-haired nonburrowing animal, and pathogenic fungi were cultured from only 3 of 243 examined. One isolate of *T. mentagrophytes* was obtained from 199 specimens collected in areas frequented by domestic animals. Of the 17 isolates of this fungus, 13 were obtained from 311 *P. polionotus*, a fluffy-furred burrowing rodent. Of these 13 isolates, 12 were obtained from specimens collected in areas frequented by domestic animals. An additional isolate was obtained from 1 of 8 *S. hispidus*, collected in similar areas. Two of 32 *P. gossypinus* from bottom land hardwoods also were infected with this fungus.

There is some evidence that rodents bearing *T. mentagrophytes* and *M. gypseum* are common in some locations and scarce in others. *Trichophyton mentagrophytes* was isolated from 16 (2.7%) of 587 animals from Baker County, and only 1 (0.4%) of 250 animals from Mitchell County. No isolates were obtained from the 157 animals from Grady, Thomas, and Dougherty Counties. In their study of commensal rats, Smith *et al.*[11] did not find infections by this fungus in 185 animals from Mitchell and Baker Counties. *Microsporum gypseum* was isolated only from specimens collected in Baker and Mitchell Counties, and seven of the 14 isolates were obtained from *P. polionotus*.

There is some indication of seasonal variation in the prevalence of the Microsporum (red variety). A study of 316 *S. hispidus*, collected in approximately equal numbers from Grady, Thomas, Baker, and Mitchell Counties from March through September, 1956, showed the following fluctuations in occurrence: 15 (28.8%) of 52 in March, 12 (26.0%) of 46 in April, 6 (14.6%) of 41 in May, 6 (14.2%) of 42 in June, 1 (1.9%) of 52 in July, 1 (2.4%) of 41 in August, and 10 (23.8%) of 42 in September. These data reveal a marked reduction in the number of isolates obtained during the hot, dry months. The factors influencing the monthly occurrence of the fungus remain unknown.

Summary

Between Oct. 13, 1954, and Sept. 30, 1956, 996 feral rodents of eight species were collected in southwestern Georgia and tested for the presence of ringworm fungi. All specimens were negative by direct microscopic and Wood's lamp examinations, and none of the animals had skin lesions. When the specimens were cultured, 114 (11.4%) yielded fungi. *Trichophyton mentagrophytes* (17), *Microsporum gypseum* (13), and Microsporum (red variety) (83) were isolated. The latter fungus has not been shown to be pathogenic to either man or lower animals. Lack of any evidence of infection other than positive cultures may indicate that the animals tested during this investigation were merely carriers of fungus elements picked up from the environment. The frequency of isolation from the various species of rodents ranged from 1.2 to 34.5 per cent.

The percentage of host animals in the various habitats increased sharply with the progression of plant succession toward pure pine. Prevalence of the fungi ranged from 6.4 per cent for animals collected in tall weeds to 47.0 per cent for those collected in pine woodlands. The number of animals collected from the various habitats diminished as plant succession progressed.

There is some evidence that *T. mentagrophytes* is most abundant in areas frequented by domestic animals and is most often found on the burrowing rodent, *Peromyscus polionotus*. The data also indicate that both *T. mentagrophytes* and *M. gypseum* are abundant in some locations and scarce or absent in others.

The rate of occurrence of the Microsporum (red variety) on *Sigmodon hispidus* ranged from 28.8 per cent in March to 1.9 per cent in July.

Microsporum gypseum was the only dermatophyte isolated from soils collected in feral rodent habitats.

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Ringworm Fungi of Large Wild Mammals in Southwestern Georgia and Northwestern Florida

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THE OCCURENCE of ringworm infections in wild animals was studied at the Emory University Field Station, Newton, Ga., as part of investigations on the ecology of diseases transmissible from animals to man. This segment of the over-all study was conducted cooperatively by the Technology and Laboratory Branches of the Communicable Disease Center. The pathogenic fungi were included in the investigations, since it has been estimated that 70 to 80 per cent of ringworm infections of man in rural areas are contracted from domestic or wild mammal sources (Georg [3]). The occurrence of ringworm fungi in feral rodents and commensal rats has been reported previously by McKeever *et al.*[4] and Smith *et al.*[5] This report is concerned with the occurrence and distribution of ringworm fungi in larger wild mammals.

MATERIALS AND METHODS

All animals were trapped with No. 2 coil-spring fox traps set at 0.1-mile intervals in lines of 125 and maintained for four nights. Ninety lines were run in three major types of habitat: pastures or cultivated fields, open pine woodland, and bottom land hardwood forest. A mixture of wildcat and fox urine, glycerin, and skunk scent was used for bait. Traps were checked each morning and the animals were brought to the laboratory in individual cages.

Using sterile forceps, hairs were plucked from the nose, forehead, middle of the back, and between the forelegs of each animal and sealed in envelopes. Each animal was examined for the presence of skin lesions.

The hair samples were tested for the presence of ringworm fungi by microscopic and Wood's lamp examinations and by culturing as described by Georg.[3] A total of 189 soil samples collected from the trapping areas was tested for the presence of keratinophilic fungi by baiting the soil with hair (Ajello [1]). All tests were performed at the Mycology Laboratory, Communicable Disease Center, Chamblee, Ga.

From the Communicable Disease Center, Bureau of State Services, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Ga., and Emory University Field Station, Newton, Ga.

The authors acknowledge the assistance of Drs. R. W. Menges and L. K. Georg and of Mr. G. W. Gorman in these studies.

For purposes of this report, a large mammal is defined as one larger than a commensal rat.

RESULTS

From Nov. 30, 1954, to Sept. 21, 1956, hair specimens from 1,758 large mammals of 14 species were tested (table 1). All microscopic and Wood's lamp examinations were negative, but *Trichophyton mentagrophytes* was cultured from 6 (0.3%) specimens; and a fungus, here designated as Microsporum (red variety), was cultured from 15 (0.8%). This latter organism is considered by Ajello [1] to be a new species; it is not known to be pathogenic. None of the animals had lesions or other clinical indications of infection.

The highest frequency of isolations was obtained from the opossum; 3.9 per cent of 436 specimens were positive. All 6 of the isolations of *T. mentagrophytes* and 11 of the 15 Microsporum (red variety) cultures were obtained from this species. Other "red variety" cultures were obtained from 2 striped skunks, 1 raccoon, and 1 cottontail rabbit.

The ecological distribution of the mammals, and of the fungi recovered, is presented (table 1). These figures were obtained by assigning all the animals from a given area to the type of habitat predominant in that area. Of the 6 opossums from which *T. mentagrophytes* was isolated, 4 were collected in pastures and cultivated fields; the other 2 were trapped in pine woodlands. Opossums were found in approximately the same numbers in the three types of habitat, but none of those from bottom land hardwoods yielded this fungus.

Of the 15 isolates of the Microsporum (red variety), 11 were obtained from animals trapped in open pine woodlands. Two isolates of this organism were recovered from animals in each of the two other habitats.

Microsporum gypseum was recovered from 13 of 189 soil samples collected in areas trapped.

TABLE 1—Ecological Distribution of Large Mammals and the Results of Tests for Ringworm Fungi Which Infect Them, in Southwestern Georgia and Northwestern Florida

Species	Habitat type			Totals	Positive for ringworm fungi (%)
	Pastures or cultivated fields	Open pine woodland	Bottom land hardwood forest		
Opossum (*Didelphis marsupialis*)	132(5)*	169(10)†	135(2)**	436(17)	3.9
Cottontail rabbit (*Sylvilagus floridanus*)	43	84(1)**	80	207(1)	0.5
Marsh rabbit (*Sylvilagus palustris*)	1	1	2	4	....
Gray squirrel (*Sciurus carolinensis*)	1	2	1	4	....
Fox squirrel (*Sciurus niger*)	18	33	8	59	....
Dog (*Canis familiaris*)	....	1	....	1	....
Red fox (*Vulpes fulva*)	4	11	5	20	....
Gray fox (*Urocyon cinereoargenteus*)	34	25	36	95	....
Raccoon (*Procyon lotor*)	199	171(1)**	213	583(1)	....
Striped skunk (*Mephitis mephitis*)	61(1)**	71(1)**	107	239(2)	0.2
Spotted skunk (*Spilogale putorius*)	3	....	3	6	0.8
Otter (*Lutra canadensis*)	....	1	....	1	....
Wildcat (*Felis rufa*)	9	31	28	68	....
Wild house cat (*Felis domestica*)	7	19	9	35	....
Totals	512(6)	619(13)	627(2)	1,758(21)	1.1

* Microsporum (red variety), 1; *Trichophyton mentagrophytes*, 4; † Microsporum (red variety), 8; *Trichophyton mentagrophytes*, 2; ** Microsporum (red variety).
The number of infected animals is indicated in parentheses.

DISCUSSION

Results of the study indicate that large wild mammals probably are of minor importance in the epidemiology of ringworm infections. Isolates were obtained from only 1.1 per cent of the animals tested, as compared with 11.4 per cent from feral rodents (McKeever *et al.*[4]) and 5.1 per cent from commensal rats (Smith *et al.*[5]). Fifteen of the 21 isolates were Microsporum (red variety), a fungus which also has been recovered from soil but which has failed to infect experimental animals. Isolates of *T. mentagrophytes*, an occasional agent of ringworm in cattle and horses (Georg[3]), were obtained from only 0.3 per cent of the wild mammals. These isolates were from opossums trapped in areas frequented by domestic animals. Since none of the animals from which dermatophytes were isolated showed any evidence of infection, the fur of the hosts may have been contaminated with spores from the environment, rather than being infected.

Opossums yielded 81 per cent of all isolates, although only 25 per cent of the animals tested were of this species. The reason for the higher rate of infection is unknown, but probably can be attributed to differences in the habits of the species tested.

SUMMARY

Between Nov. 30, 1954, and Sept. 21, 1956, hair specimens from 1,758 large wild mammals from southwestern Georgia and northwestern Florida were tested for the presence of ringworm fungi. All specimens were negative by microscopic and Wood's lamp examinations. Isolates were obtained from 21 (1.1%); six of the isolates were *Trichophyton mentagrophytes* and 15 were Microsporum (red variety). The latter organism is not known to be pathogenic.

All isolates of *T. mentagrophytes* were obtained from opossums collected in areas frequented by domestic animals. Eleven of the 15 isolates of Microsporum (red variety) were from animals collected in open pine woodlands. This organism was

isolated from 11 opossums, 1 cottontail rabbit, 1 raccoon, and two striped skunks.

Lack of any evidence of infection in animals from which dermatophytes were isolated indicates that the fur of these animals may have been contaminated with spores from the environment, rather than being infected. *Microsporum gypseum* was recovered from 13 of 189 soil samples from areas trapped.

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Survival of *Brucella Abortus*, *Leptospira Pomona*, and *Vibrio Fetus* in the Illini Variable Temperature Semen Extender at 29 C.

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SINCE THE INTRODUCTION of artificial insemination of cattle, the possibility of transmitting infectious organisms via the semen has been investigated widely.

The presence of *Brucella abortus* in semen from infected bulls and the spread of the disease through artificial insemination have been shown.[2, 6, 13] The prevalence of *Vibrio fetus* infection among bulls and the possibility of transmission of this disease by artificial insemination also have been demonstrated.[6, 9, 15] It is well known that chronic bovine leptospirosis is characterized by kidney infection and the shedding of leptospiras in the urine.[2] Although the possibility of carrier bulls shedding the organism into the semen is reasonable, no report of the occurrence of *Leptospira pomona* in bovine semen has been found.

Efforts usually are made to eliminate infected bulls from the artificial breeding studs. Attempts to control the spread of disease by infected semen during artificial insemination have been made by adding antibacterial agents to the semen diluents.[9, 12] However, a recent report has shown that penicillin and streptomycin added to semen diluent are effective against *V. fetus* only if a minimum of six hours' exposure at 5 C. is allowed.[10] The addition of glycerol to diluted semen also has been reported to limit the effectiveness of antibiotics against *V. fetus*.[5] Studies of *L. pomona* in a diluent containing sulfanilamide, penicillin, and streptomycin have shown that this organism will remain viable during storage at 5 C. for one week.[4]

Recently, VanDemark and Sharma [14] reported the successful maintenance of fertility of bull semen for six days at room temperature by storing semen in sealed ampules under carbon dioxide in a new diluent called the Illini variable temperature (IVT) diluent.

This report concerns the survival of *Br. abortus*, *L. pomona*, and *V. fetus* in the IVT diluent at 29 C.

MATERIALS AND METHODS

The IVT semen diluent was prepared in the manner reported by VanDemark and Sharma.[14] The diluent was composed of 20.0 Gm. of sodium citrate dihydrate, 2.1 Gm. of sodium bicarbonate, 0.4 Gm. of potassium chloride, 3.0 Gm. of glucose, and 3.0 Gm. of sulfanilamide dissolved (by heating to near boiling) in a liter of water distilled over glass. This solution then was cooled to room temperature and saturated with carbon dioxide by bubbling the gas through it for ten minutes (or until the pH was reduced to about 6.35). After saturation with carbon dioxide, enough fresh egg yolk was added to make a diluent consisting of 10 per cent yolk. Penicillin in a final concentration of 1,000 I.U. per milliliter and dihydrostreptomycin sulfate in a final concentration of 1,000 μg. per milliliter were added to one portion. A second portion was treated in the same way, except that the final concentration of penicillin was 500 I.U. per milliliter and that of dihydrostreptomycin sulfate was 500 μg. per milliliter. Sulfanilamide and the antibiotics were omitted from a third control portion. Semen was diluted so that each milliliter contained approximately 25 million spermatozoa.

High and low antibiotic levels of diluted semen were inoculated with *Br. abortus*, *L. pomona*, and *V. fetus*. Forty-eight hour tryptose agar slants of *Br. abortus* strain 2308 incubated in air at 37 C. were washed off with sterile saline solution and adjusted to a concentration of organisms equivalent to the turbidity of McFarland's nephelometer tube No. 1. Tryptose agar slants of *V. fetus* strain K60453 were treated in the same way as *Br. abortus*, except that they were incubated under approximately 10 per cent carbon dioxide. *Leptospira pomona* strain 16-P54874 was grown for five days in Stuart's medium. Before inoculation of the diluted semen, part of the culture was diluted 1:10 with 0.5 per cent formolized saline solution to arrest motility, and the number of leptospiras present was determined by a Petroff-Hausser counting chamber.

Brucella abortus, *L. pomona*, and *V. fetus* were inoculated into high and low antibiotic levels of diluted semen and into a control of the diluent without antibiotics and sulfanilamide in final concentrations of 100,000 organisms per milliliter. One milliliter of each, in triplicate, was transferred by syringe and needle to glass ampules (2.2-ml. total capacity) and sealed. Immediately after sealing and after two days' and four days' incubation at 29 C., the ampules were opened and the entire contents of each ampule were transferred into test

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tubes containing 5 ml. of sterile saline solution. The tubes were centrifuged at 3,000 r.p.m. for 30 minutes and subcultured to determine whether viable organisms were present.

The sediment of each tube containing *L. pomona* was transferred to 3 ml. of Stuart's medium and incubated at 29 C. Each tube was subcultured into Stuart's medium after seven days' incubation. Tubes were examined at seven-day intervals for three weeks by dark-field illumination for motile, viable organisms.

A loop of the sediment of each tube containing *Br. abortus* or *V. fetus* was streaked on a tryptose crystal violet agar plate. The remaining portion of the sediment was transferred to 6 ml. of thiol broth (Difco), in the case of *V. fetus*, and 6 ml. of tryptose broth in the case of *Br. abortus*. Tubes of broth were subcultured at three and six days on tryptose crystal violet agar. Cultures of *Br. abortus* were incubated in air at 37 C., while cultures of *V. fetus* were incubated under approximately 10 per cent carbon dioxide at 37 C.

A third portion of the diluent without antibiotics and sulfanilamide served as control material. Separate portions were inoculated with *Br. abortus*, *L. pomona*, and *V. fetus*. The ampules were filled and examined for viable organisms in the same way as the groups containing antibiotics.

RESULTS

Brucella abortus, *L. pomona*, and *V. fetus* were not viable in diluted bovine semen in any replicate at high or low levels of antibiotics when examined after 0, 48, and 96 hours' incubation at 29 C. *Leptospira pomona* was viable in control replicates at 0 hours but not at 48 and 96 hours. *Brucella abortus* and *V. fetus* were viable in all control ampules. The suspension of each organism used for inoculation was cultured and found to be viable.

A culture of *L. pomona* (56,000,000/ml.) in Stuart's medium was diluted 1:2, 1:5, $1:10^1$, $1:10^2$, $1:10^3$, and 10^4 with the semen diluent without sulfanilamide, antibiotics, and semen to determine the effect of the diluent alone on *L. pomona*. The tubes were incubated at 29 C. for seven days and subcultured in Stuart's medium. Leptospiras were observed by dark-field illumination in all tubes immediately after preparation of dilutions but, after 24 hours' incubation, they were observed only in the undiluted Stuart's medium. Inoculation of guinea pigs with the subculture of the 1:2 dilution failed to reveal the presence of leptospiras.

DISCUSSION

Since *Br. abortus*, *L. pomona*, and *V. fetus* are the primary infectious agents of bovine reproductive diseases, the survival of these organisms in the IVT diluent at 29 C. was investigated. As was found by Bryan and Boley [4] with other diluents, *L. pomona* survived for only one day in the IVT diluent, even in the absence of sulfanilamide, dihydrostreptomycin sulfate, and penicillin. *Brucella abortus* and *V. fetus* were less sensitive; they survived in plain IVT for 96 hours, but were not viable at 0 hours when sulfanilamide, dihydrostreptomycin sulfate, and penicillin were added.

It is apparent that the temperature at which the diluted semen containing antibiotics is held will influence the survival of organisms. The general practice has been to hold diluted semen at refrigerator temperature (approx. 5 C.) or to freeze and store it at −79 C. Bryan and Boley [4] reported that *L. pomona* survived for seven days in mediums containing penicillin or streptomycin when stored at 5 C., but for only one or two days at 28 C. Several reports have indicated that *V. fetus* in diluted bovine semen will survive storage at 5 C. in the presence of antibiotics.[1, 7, 10, 11] At 30 C. or 37 C., the antibiotics are more active against this organism.[11] Thus, it appears that the storage of bull semen at room temperature in the IVT diluent with sulfanilamide and antibiotics should effectively prevent the danger of spreading brucellosis, leptospirosis, or vibriosis, since the causative organisms did not survive after contact with the IVT diluent.

SUMMARY

1) Survival of *Brucella abortus* strain 2308, *Leptospira pomona* strain 16-P54874, and *Vibrio fetus* strain K60453 inoculated into semen diluted with the Illini variable temperature (IVT) diluent and sealed in glass ampules was tested at 29 C.

2) Separate ampules inoculated with 100,000 organisms per milliliter were opened and cultured after 0, 48, and 96 hours of storage.

3) Control ampules contained IVT diluent without sulfanilamide and antibiotics. *Leptospira pomona* was viable only at the 0 time; *Br. abortus* and *V. fetus* were viable at 0, 48, and 96 hours of storage.

4) Viable *Br. abortus*, *L. pomona*, and *V. fetus* could not be demonstrated in the IVT diluent containing 0.003 Gm. of sul-

fanilamide, 500 I.U. of penicillin, and 500 μg. of dihydrostreptomycin sulfate per milliliter after 0, 48, and 96 hours of storage.

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Detection of Foot-and-Mouth Disease Antibody in Vaccinated Cattle by Direct Complement Fixation

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By use of a direct complement (C′) fixation technique, specific antibody may be detected in serums from cattle experimentally infected with foot-and-mouth disease virus (FMDV).[3] In this report, the C′-fixation technique has been applied to study the appearance and disappearance of antibody in serums of cattle inoculated with monovalent foot-and-mouth disease (FMD) vaccines, of revaccinated cattle, and of cattle receiving viable FMDV at 21 and 75 days after primary vaccination.

Materials and Methods

Direct Complement-Fixation Test.—The technique of C′ fixation and the standardization of the reagents have been described in a previous paper.[3]

Antiserums.—Healthy adult Swedish cattle, free from previous exposure to FMDV, were used in these trials. After taking pre-exposure blood samples, the animals were inoculated subcutaneously with 15 ml. of monovalent FMD vaccine prepared at Lindholm by the Schmidt-Waldmann method for general field use.[4,5] The animals were housed on the mainland pending completion of serological tests. Challenge inoculations were made on the day of transfer from the mainland to isolation units on Lindholm Island. Approximately 10^4 infective doses (bovine) of homologous type FMDV were injected intradermolingually. All blood samples were taken from the jugular veins. The separated serum was heated for 30 minutes at 56 C. and stored at approximately −40 C. in an electric deep-freeze.

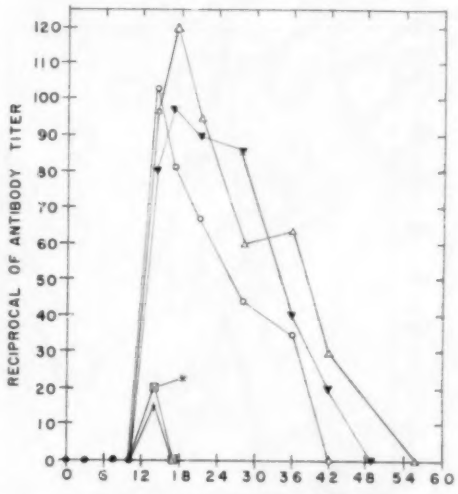
Antigen.—The antigen used in the C′-fixation analyses was a 1:60 veronal buffer dilution of pooled FMD vesicle fluid collected from 20 to 30 cattle. It was treated and stored as described in a previous paper.[3]

Results

Experiment 1

Antibody Response to Primary Vaccination with Type A, Strain A_5, Foot-and-Mouth Disease Vaccine.—To determine the usefulness of the direct method of C′ fixation for detection of C′-fixing antibody in vaccinated cattle, 6 Swedish cows (No. 17 to 22) were vaccinated with monovalent A_5 vaccine. During the test period, it was essential to preclude exposure to FMDV, as it was desired to measure only antibody produced as a result of the vaccination. Two nonvaccinated Swedish cows (No. 23 and 24) served as controls and were stabled and managed in the same manner as the test animals.

Results of the C′-fixation analyses obtained with serums from 6 vaccinated Swedish cattle are shown (graph 1). Anti-



Circles = cow 17; triangles = cow 18; dagger = cow 19; darkened triangles = cow 20; squares = cow 21; crosses = cow 22; darkened circles signify that all blood samples taken on this day were negative for C′-fixing activity; asterisk = data for cows 21 and 22 were identical.

Graph 1—Course of antibody appearance and disappearance in Swedish cattle inoculated with type A_5 Schmidt-Waldmann foot-and-mouth disease vaccine. Serums from samples obtained on the days shown were tested for antibody by direct complement fixation. All serum samples taken after 14 days for cows 19, 21, and 22, after 35 days for cow 17, and after 42 days for cow 20 were negative for direct complement-fixation activity.

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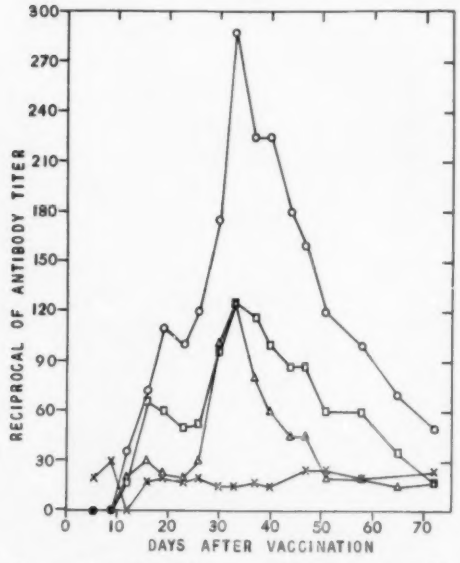
body was detected in serum from all of the vaccinated animals at some time after vaccination. Serums from 2 nonvaccinated controls remained negative for C′-fixing antibody during the entire 56-day test period.

Individual responses of the animals differed with respect to quantity of antibody produced, as measured by C′ fixation, and the length of time antibody was detectable following vaccination. Direct complement-fixing antibody was found in serums from animals 19, 21, and 22 only in the samples taken 14 days after vaccination. In these animals, the titers were low and may not be considered significant. However, the conclusion that the C′-fixing activity of these serums is due to production of FMD antibody resulting from vaccination is strongly indicated; first, antibody was detected on the fourteenth day after vaccination, which is the time most vaccinated animals showed peak or near-peak antibody titers and, second, all other serums from these animals were negative for antibody. It will be shown later that serums from nonexposed cattle which show C′-fixing activity retain this reactivity at a consistently low level over a long period of time.



Squares = cow 25; circles = cow 26; triangles = cow 27; crosses = cow 28; darkened circles signify that all blood samples taken from the vaccinated cattle on this day were negative for C′-fixing activity.

Graph 2—Course of antibody appearance and disappearance in Swedish cattle inoculated with type A_5 Schmidt-Waldmann foot-and-mouth disease vaccine and revaccinated 23 days later. Serums from blood samples obtained on the days shown were tested for antibody by direct complement fixation.

EXPERIMENT 2

Antibody Response to Revaccination with A_5 Vaccine 23 Days After Primary Vaccination.—To determine the effect of a second dose of vaccine on the C′-fixing antibody titer, 3 Swedish cattle (No. 25, 26, and 27) were injected with A_5 FMD vaccine a second time, 23 days after the first vaccination. A nonvaccinated control (No. 28) was housed with the test animals throughout the experimental period, and blood samples were taken at the same time as those from the test group. Results of the C′-fixation analyses with serums from this group of animals are given (graph 2).

Antibody was first demonstrable in serum samples from cattle of this group 12 days after primary vaccination. These results are in close agreement with data obtained in the first experiment and indicate that the lag period, *i.e.*, the time during which antibody is not detectable by this test, is longer following vaccination than infection. The C′-fixing antibody titers, which were at a maximum 16 to 19 days after primary vaccination, were beginning to descend at the time of revaccination.

The response to the second vaccination resembles a typical anamnestic reaction in that antibody appears more quickly and reaches a higher titer than it does following primary vaccination. In this experiment, there was a demonstrable antibody increase in three days and the titers were highest ten days after revaccination.

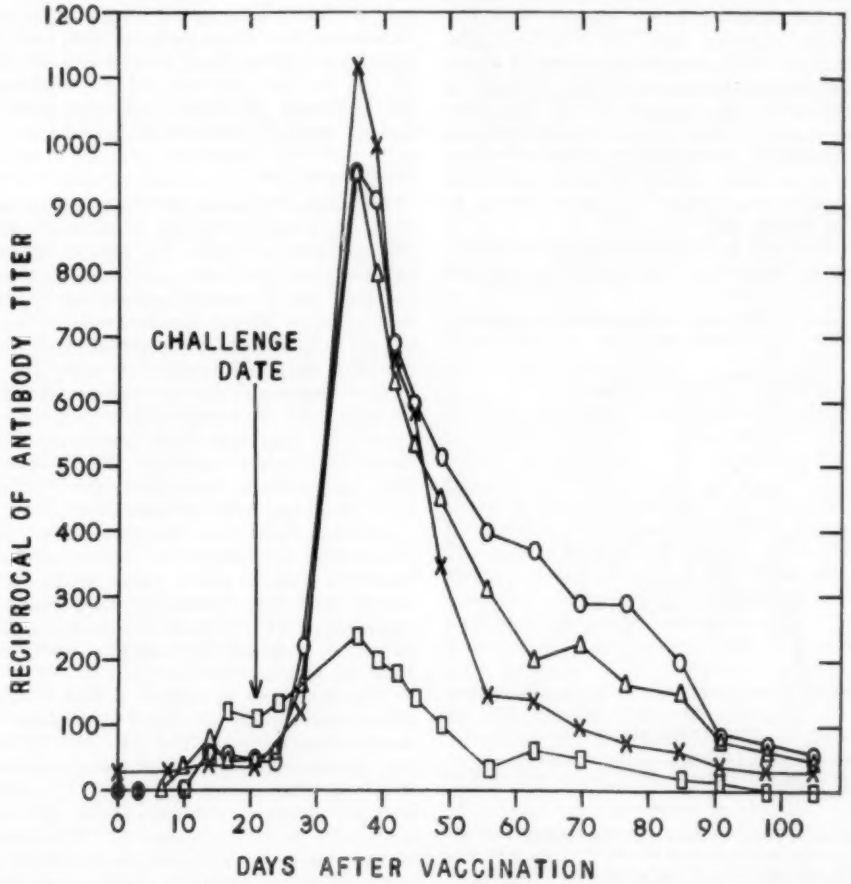
With the exception of the serum obtained 14 days after vaccination, all serum samples from nonvaccinated cow 28 showed C′-fixing activity. The reactivity differed from that observed in serums from vaccinated or infected cattle, since it remained at a relatively constant low level throughout the entire 72-day test period. This aberrant behavior indicates that the C′-fixing reactivity associated with serum samples from animal 28 was probably not due to FMDV antibody. To distinguish this type of reaction from that encountered in vaccinated and infected cattle, it is referred to in this paper as "normal" C′-fixing reactivity.

EXPERIMENT 3

Antibody Response to Challenge with Homotypic Foot-and-Mouth Disease Virus 21 Days After Primary Vaccination.—In the previous experiment, it was shown that C′-fixing antibody titers increased in serums from cattle which were revaccinated 23 days after primary vaccination. This experiment was set up to determine antibody response in cattle challenged with active virus 21 days after vaccination, at the time when the antibody titers are at or near maximum levels.

Three Swedish cows (No. 29, 30, and 31) were vaccinated with 15 ml. of type A_5 FMD vaccine. On the twenty-first day after vaccination, these animals, along with a nonvaccinated control (No. 32), were taken to Lindholm Island and challenged with homotypic FMDV. Serum samples were taken at semiweekly or weekly intervals for 84 days after challenge and tested for antibody by C′ fixation (graph 3).

In this group, animal 30 first showed antibody on the tenth day after vaccination, whereas antibody was not detectable



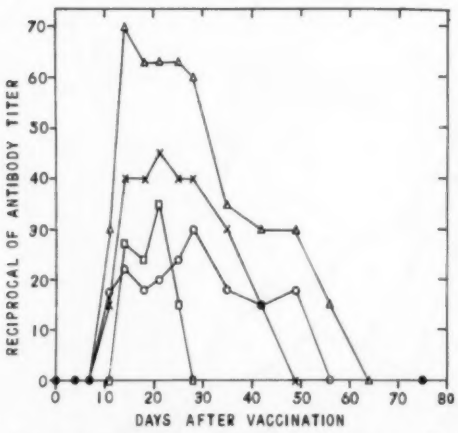
Circles = cow 29; triangles = cow 30; squares = cow 31; crosses = cow 32; darkened circles signify that all blood samples taken from the vaccinated cattle on this day were negative for C′-fixing activity.

Graph 3—Course of antibody appearance and disappearance in Swedish cattle vaccinated with type A_5 Schmidt-Waldmann foot-and-mouth disease vaccine and challenged 21 days later by intradermolingual inoculation of active type A_5 foot-and-mouth disease virus. Serums obtained on the days shown were tested for antibody by direct complement fixation.

in serum from cows 29 and 31 until the fourteenth day. As expected, the vaccinated cattle withstood the challenge inoculum of virus and the nonvaccinated control showed generalized infection involving the feet.

At the time of challenge, antibody titers resulting from vaccination had passed their maximum levels and were beginning to descend. Inoculation of active virus resulted in an increase of circulating antibody, which rose to a maximum in 14 days. By comparison of these results with those of the previous experiment, it appears that active virus inoculated into vaccinated cattle produced a much greater C′-fixing antibody response than did a second dose of vaccine. This might be expected since, within certain limits, antibody response is governed by the magnitude of the antigenic stimulus and, in vaccinated animals inoculated on the tongue with active virus, there is a short period of local proliferation which is indicated by small lesions at site of injection.

In cow 31, the antibody response to the challenge inoculum was far below that observed in the other animals in this group. This may be a reflection of the fact that this animal had a higher C′-fixing antibody titer at time of challenge, which may mean that it was better prepared to combat the local proliferation of virus. Such a state would presumably result in a diminished antigenic stimulus in relation to the other cattle in this group. This might be the reason for the lower antibody level.



Squares = cow 33; circles = cow 34; triangles = cow 35; crosses = cow 36; darkened circles signify that all blood samples taken on this day were negative for C′-fixing activity.

Graph 4—Course of antibody appearance and disappearance in Swedish cattle vaccinated with type O_2 Schmidt-Waldmann foot-and-mouth disease vaccine. Serums from blood samples obtained on the days shown were tested for antibody by direct complement fixation. Serum samples taken after 25 days from cow 33, after 42 days from cow 35, after 49 days from cow 34, and after 56 days from cow 35 were negative for direct complement-fixing activity.

The prechallenge blood samples from nonvaccinated cow 32 showed a low level of C′-fixing activity similar to that observed in serums from cow 28. It is of interest that, at 21 days, the level of normal C′-fixing activity of serum from cow 32 was about the same as the C′-fixing activity resulting from vaccination of cows 29 and 30; yet, cow 32 did not withstand the challenge inoculum, whereas cows 29 and 30 were refractory to infection.

EXPERIMENT 4

Antibody Response to Vaccination with Type O_2 Foot-and-Mouth Disease Virus.—The 4 Swedish cattle (No. 33, 34, 35, and 36) used in this test series were each vaccinated with 15 ml. of monovalent type O_2 FMD vaccine. Blood samples were taken at semiweekly or weekly intervals for 75 days. At that time, C′-fixing antibody was no longer demonstrable in serums of these animals, and they were taken to Lindholm Island for challenge with homotypic FMDV. Seven days after challenge, blood samples were again taken and tested for antibody by C′-fixation analysis (graph 4).

Pre-exposure blood samples from these cattle were negative for C′-fixing antibody. Samples taken after vaccination were tested, and the curves relating antibody appearance with respect to time are similar to those obtained in animals vaccinated with A_5 vaccine.

The challenge inoculum, given 75 days after vaccination, produced generalized infection involving the feet in all of the cattle. After challenge, there was an increase in circulating antibody (table 1) similar to that previously observed in the tests with type A_5 virus (graph 3). This experiment represents an example in which absence of C′-fixing antibody in vaccinated cattle ran parallel to absence of resistance to FMDV.

To determine that infection was due to type O_2 virus, and not to a contamination with FMDV of another type, each of the

postchallenge serums was assayed for C′-fixing activity with types A_5 and C_1 FMDV. The results of these tests (table 1) demonstrate that the antibody response was greatest to type O_2 virus, indicating that the infection was due to virus of this type.

DISCUSSION

The results of this study show that, by C′ fixation, it is possible to detect FMDV antibody in serums from cattle inoculated with Schmidt-Waldmann FMD vaccine. In these experiments, antibody was usually first detectable ten to 14 days after vaccination. It rose to a peak titer in 14 to 23 days, dropped off precipitously at first, then more slowly, and could be detected in some cattle for as long as 56 days after vaccination.

The quantity of antibody produced, as measured by C′-fixing activity, was much less in vaccinated animals than in cattle which were given active virus.[3] This would be expected, since multiplication of active virus introduced into the host represents a massive antigenic stimulus exceeding that induced by vaccine. Another major difference in antibody response of actively infected cattle, as compared with those given vaccine, concerns the length of the lag period after inoculation of antigen during which no antibody can be demonstrated. In the vaccinated animals in these experiments, antibody first appeared ten to 14 days after vaccination. However, antibody nearly always was found in experimentally infected cattle seven days after infection.[3]

Normal serums from 2 of 36 cattle examined gave C′ fixation with type A_5 FMDV. Since the level of reactivity of these serums remained relatively constant over a long period of observation, it differs from the C′-fixing activity found in serums from infected or vaccinated cattle, in which the C′-fixing antibody titers are either increasing or decreasing. In this paper, the activity associated with normal cow serum has been termed "normal C′-fixing reactivity," for purposes of differentiation. Since a crude antigen preparation was used, this C′-fixing activity may be due to a reaction (or reactions) of these serums with components of vesicle lymph other than FMDV.

General experience in FMD epizooties indicates that type-specific resistance to reinfection lasts about 12 months.[1] Immunity conferred by FMD vaccination is reported to last approximately four months.[2] These experiments were not devised to determine the possible relationship between presence of C′-fixing antibody and resistance of animals to infection. Nevertheless, evidence of some correlation may be obtained from data reported here. In experiment 3, vaccinated animals showing C′-fixing antibody were resistant at the time of challenge and, in experiment 4, vaccinated animals in which C′-fixing antibody was no longer demonstrable were susceptible to experimental infection.

TABLE 1—Results of Type O_2 Foot-and-Mouth Disease Challenge in Four Swedish Cattle* and of Direct Complement-Fixation Analyses on Serums Taken from These Cattle Seven Days After Challenge

Item	Cow No. 33	34	35	36
Reciprocal of antibody titer to type O_2 FMDV 75 days after vaccination	0	0	0	0
No. feet on which secondary FMD lesions were observed after challenge	1	1	1	2
Reciprocal of antibody titer 7 days after challenge				
with type O_2 FMDV	925	975	250	1600
with type A_5 FMDV	30	100	24	70
with type C_1 FMDV	0	100	20	40

* Vaccinated 75 days previously with type O_2 Schmidt-Waldmann foot-and-mouth disease vaccine.

It is not unreasonable to assume that serums obtained from naturally infected animals and cattle vaccinated in the field would yield C′-fixation results similar to those reported here and elsewhere.[3] If this is so, it would seem unlikely, in view of the field experiences cited, that absence of C′-fixing antibody in cattle would be definitive proof of susceptibility to natural infection. However, the length of time that C′-fixing antibody has been shown to be demonstrable in serums from experimentally infected and vaccinated cattle falls within the periods reported for immunity resulting from natural infection or field vaccination. It should follow that the presence of C′-fixing antibody would be an indication of resistance to natural FMDV infection. In any study to explore this, it would be necessary to show that C′-fixing activity found in cattle serums is not due to nonspecific C′-fixing reactivity. The reason for this is shown in experiment 3, in which serums from 1 nonvaccinated cow and 2 vaccinated cattle had approximately the same C′-fixing activity; yet, the nonvaccinated animal was susceptible to gen-

eralized infection and the 2 vaccinated animals were not.

SUMMARY

1) By direct complement (C′) fixation, antibodies have been detected in serums from cattle inoculated with type A_5 and type O_2 Schmidt-Waldmann foot-and-mouth disease vaccines.

2) Maximal C′-fixing antibody titers were obtained two to three weeks after primary vaccination.

3) Revaccination in 23 days resulted in an increased production of antibody which exceeded that following primary vaccination.

4) Inoculation of active virus, 21 days after vaccination, elicited an antibody response similar to that observed in nonvaccinated cattle, although vaccinated animals were resistant to generalized infection at this time.

5) A relationship between presence of C′-fixing antibody and resistance to foot-and-mouth disease infection has been postulated.

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Comparative Potencies of Tuberculins Produced on Asparagine and Ammonium Glutamate Mediums

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FOLLOWING a report by Henley,[6] in 1929, on the use of dextrose in synthetic mediums for growing tubercle bacilli, Dorset and Henley,[5] in 1934, made available details on a synthetic medium and its use in a procedure for routine production of tuberculin derived from *Mycobacterium tuberculosis* var. *hominis*, strains PN, DT, and C.

Also in 1934, Dorset [3, 4] reported on comparative tests, on more than 20,000 cattle, of the synthetic-medium tuberculin and Koch's old tuberculin derived from the same strains. In one series of tests with 13,288 cattle, 1,127 reacted to both types of tuberculin and 141 to the synthetic-medium tuberculin only. Of the 1,127 that reacted to both, 1,070 (94.9%) had lesions; of the other 141 reactors, 135 (95.7%) had lesions. In these tests, the synthetic-medium tuberculin was demonstrated to be an improvement over Koch's old tuberculin, and the synthetic medium was used routinely without interruption until World War II, using asparagine as the source of nitrogen.

About 1944, the less expensive ammonium glutamate was substituted for asparagine as a source of nitrogen [14, 15] in certain batches of medium. Henley and LeDuc [8] had reported, in 1939, that ammonium glutamate afforded as much dry weight of tubercle bacilli when used as the source of nitrogen as did asparagine. Furthermore, they found that tuberculins derived from the two sources showed no detectable differences in potency when compared on 4 sensitized guinea pigs.

At first, small batches of glutamic acid were used to produce the ammonium glutamate medium, and it was found that complete uniformity and adequacy of growth was not always obtained with certain brands of glutamic acid. Henley [7] reported that the addition of zinc ion was necessary for adequate growth of the tubercle bacillus in a medium prepared from certain lots of malic acid, used in the form of ammonium malate as the source of nitrogen. But, in this work, when zinc was added to the ammonium glutamate medium, lack of uniformity and inadequacy of growth did not decrease. Furthermore, after the war (1948), certain brands of asparagine were found to be inadequate as nitrogen sources, even with the addition of zinc ion.

Baisden [1] reported, in 1951, that in addition to the elements known to be present in the synthetic medium, the following were detectable by spectroscopic analysis of the ash of tubercle bacilli grown on medium using satisfactory brands of asparagine: aluminum, barium, boron, calcium, cobalt, copper, lead, manganese, molybdenum, nickel, silver, sodium, strontium, and zinc. The addition of traces of these elements to unsatisfactory asparagine medium and to ammonium glutamate medium afforded consistently adequate growth. Subsequent work which dealt with the simplification of the mixture of supplements has shown that 1.4 mg. of anhydrous copper sulfate and 14.0 mg. of hydrated zinc sulfate per liter of medium will suffice for the consistent production of adequate growth of the previously mentioned strains of *M. tuberculosis* on both asparagine and ammonium glutamate mediums. From 1950 until 1954, about one half of the tuberculin produced for field use in the government testing program was derived from ammonium glutamate medium and one half from asparagine medium, each of which contained the mineral supplements. After 1954, the tuberculin produced in the government laboratory was derived from asparagine medium.

In view of the extensive use of ammonium glutamate medium during the war and postwar period, it seemed desirable to publish the experimental work on which the confident use of tuberculins derived from this source was based. This report concerns such experimental work, carried out in 1946. Satisfactory results in the field have confirmed the experimental results.

EXPERIMENTAL PROCEDURE AND RESULTS

In the course of this work, four experiments were carried out in which tuberculins derived from asparagine and ammonium glutamate mediums were compared for potency.

Asparagine Medium.—The composition of the asparagine medium [5, 6] is as follows:

L(−)asparagine	14.00 Gm.
$K_2HPO_4 \cdot 3H_2O$ (C.P. grade)	1.80 Gm.
Sodium citrate, $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}H_2O$, C.P.	0.90 Gm.
$MgSO_4 \cdot 7H_2O$, C.P.	1.50 Gm.
Ferric citrate, scales	0.30 Gm.

From the Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. The research was conducted at Beltsville, Md. (Johnson), Auburn, Ala. (Larsen and Groth), and Washington, D.C. (Baisden).

Dextrose, C.P. or U.S.P.	10.00 Gm.
Glycerol, C.P. or U.S.P.	100.00 Gm.
Distilled water, q.s.	1,000.00 ml.

The medium is autoclaved at 116 to 118 C. for 25 minutes in 100-ml. amounts in 300-ml. Erlenmeyer flasks, the temperature being lowered as rapidly as possible after sterilization.

Ammonium Glutamate Medium.—The composition of the ammonium glutamate medium [2, 14, 15] is as follows:

L(+)glutamic acid, at least 99.5 per cent pure	12.50 Gm.
Ammonium hydroxide, C.P., sufficient to bring suspension of glutamic acid into solution having pH of 7.2, usually slightly more than	5.90 ml.
$K_2HPO_4 \cdot 3H_2O$, C.P.	1.80 Gm.
Sodium citrate, $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}H_2O$, C.P.	1.68 Gm.
$MgSO_4 \cdot 7H_2O$, C.P.	1.50 Gm.
Ferric citrate, scales	0.30 Gm.
Dextrose, C.P. or U.S.P.	10.00 Gm.
Glycerol, C.P. or U.S.P.	100.00 Gm.
Distilled water, q.s.	1,000.00 ml.

Glutamic acid is suspended in about 50 ml. of distilled water, and the pH is adjusted with ammonium hydroxide. The method of sterilization is the same for both mediums. After sterilization, this medium contains an amount of nitrogen equivalent to that in finished asparagine medium.

Supplements.—These supplements are added in solution form after the addition of the other ingredients to the mediums:

$CuSO_4 \cdot 5H_2O$, C.P.	0.0014 Gm.
$ZnSO_4 \cdot 7H_2O$, C.P.	0.0140 Gm.

Experiment 1.—Ten samples of tuberculins produced by the method described by Dorset and Henley [5] were tested; five were derived from asparagine medium and five of approximately equal age from ammonium glutamate medium. Each sample of tuberculin was derived from a different batch of medium. The five batches of asparagine medium were 24 liters each, and the five batches of ammonium glutamate medium were 12 liters each. As stated earlier, the initial work on ammonium glutamate medium was carried out on small batches. Each lot of medium was seeded from approximately equal numbers of seed cultures of each of the strains, PN, DT, and C, so that each of the samples of the asparagine tuberculin and of the ammonium glutamate tuberculin represented a mixture of the culture filtrates from 80 and 40 cultures, respectively, of each strain.

Doses of 0.2 ml. each of 10-, 100-, and 1,000-fold dilutions of each of the samples of tuberculin were injected intradermally in triplicate on each of 5 cattle sensitized by the intraperitoneal instillation of 3 Gm. of heat-killed *Mycobacterium tuberculosis* var. *bovis*, strain 854, according to the method of Johnson.[9] These doses also were injected in each of 5 cattle similarly sensitized to heat-killed *Mycobacterium paratuberculosis*.

The sites of placement [9] of the injections of the 15 solutions of asparagine tuberculin were the same on each animal, 45 injections at least 3 inches apart being made on the right side of each animal. Three rows of 12 sites each covered an area from the neck to the hip, one row of six sites was placed on the lower part of the side, and one row of three sites still lower in the center of the side. Three replications of asparagine-1 (A-1) were injected into the first nine sites of the upper row and one replication of A-2 into the last three sites, each replication consisting of three injections of 10-, 100-, and 1,000-fold dilutions, respectively. Two replications of A-2 and two replications of A-3

TABLE 1—Comparison of Asparagine and Ammonium Glutamate Tuberculins on Five Cattle Sensitized to Mycobacterium Tuberculosis var. Bovis and Five Sensitized to Mycobacterium Paratuberculosis

Type*	Av. on *M. tuberculosis* var. *bovis*†			Av. on *M. paratuberculosis*†		
	1/10	1/100	1/1000	1/10	1/100	1/1000
Asparagine-1	6.28	3.28	1.22	3.22	1.40	0.68
Ammonium glutamate-1	7.98	4.50	3.00	3.58	1.65	0.62
Asparagine-2	7.80	3.67	1.33	3.95	1.72	0.70
Ammonium glutamate-2	8.72	4.38	1.47	5.10	2.30	0.65
Asparagine-3	7.67	4.25	1.55	3.95	2.02	0.62
Ammonium glutamate-3	8.70	5.05	1.82	5.38	2.33	0.73
Asparagine-4	8.60	4.20	1.70	4.43	1.87	0.62
Ammonium glutamate-4	9.52	4.87	1.58	5.27	1.83	0.52
Asparagine-5	7.95	3.40	1.10	4.06	2.44	0.63
Ammonium glutamate-5	7.97	3.80	1.37	5.16	2.26	0.70

* The asparagine tuberculin of each pair was tested on the right side of an animal and the ammonium glutamate tuberculin on the left side, the sites of the injections being as identical as possible. † Average of three replications of each tuberculin per animal; reactions are given in millimeters of increase in skin thickness.

TABLE 2—Comparison of Asparagine and Ammonium Glutamate Tuberculins on Five Cattle Sensitized to Mycobacterium Tuberculosis var. Bovis and Five Sensitized to Mycobacterium Paratuberculosis

	Av. on *M. tuberculosis* var. *bovis*†			Av. on *M. paratuberculosis*†		
Type*	1/12.5	1/125	1/1250	1/12.5	1/125	1/1250
Asparagine	5.77	3.33	1.25	4.31	2.30	0.95
Ammonium glutamate	6.75	4.10	1.60	4.68	2.49	0.94

* Glycerol:nitrogen ratio varied for each type of medium. Placement of a pair of tuberculins, one of each type, having the same glycerol:nitrogen ratio, was not in corresponding sites on the two sides of an individual animal. † Average of three replications of each tuberculin, five tuberculins of each type, on each animal, given in millimeters of increase in the skin thickness.

were injected into the next row of 12 sites. One replication of A-3 and all three replications of A-4 were injected into the lowest row of 12 sites. Two replications of A-5 were injected into the row of six sites and the last replication of A-5 was injected into the row of three sites. The same procedure was used for testing solutions of ammonium glutamate tuberculin, except that the placements were on the left sides of the animals. Thus, an asparagine tuberculin and an ammonium glutamate tuberculin of approximately equal age were compared in sites which were as identical as possible on opposite sides of each animal.

Skin thickness measurements were made at the sites of injections before and 48 hours after the injections, using the dermal thickness gauge described by Kummer and Johnson.[11] Increases in skin thickness were compared for each pair of tuberculins by statistical methods indicated by Wadley.[16,17] Since there were five pairs of tuberculins, five separate analyses were made of the data obtained on each sensitization.

The average increases in skin thickness resulting from the tests of the tuberculins in this experiment are given (table 1).

Experiment 2.—Filtrates of cultures of the previously mentioned strains of *M. tuberculosis* grown on asparagine and on ammonium glutamate mediums prepared with various glycerol:nitrogen ratios were tested on cattle sensitized in the same way as in experiment 1. Five pairs of tuberculins were tested, the asparagine and the ammonium glutamate tuberculins of each pair having been grown on mediums with approximately the same glycerol:nitrogen ratio. The amount of growth produced varied according to this ratio; therefore, the culture filtrates were adjusted on the basis of dry weight of organisms produced. The 0.2-ml. dose of the highest concentration of each tuberculin tested was the equivalent of 0.64 mg. of dry weight. The highest concentration was considered to be equivalent to a 12.5-fold dilution of tuberculin, derived from the culture filtrate by the regular method. Two other dilutions were tenfold each.

Four of the pairs of tuberculins were each derived from eight cultures of each strain, while the individual tuberculins of the fifth pair were derived from 80 asparagine cultures of each strain and 40 ammonium glutamate cultures of each strain, respectively. Each pair was replicated three times on each of 5 cattle sensitized to *M. tuberculosis* var. *bovis* and on 5 sensitized to *M. paratuberculosis*. The replications of asparagine-1 (A-1), ammonium glutamate-1 (G-1), A-2, G-2, and A-3 were placed on the right side, and those of G-3, A-4, G-4, A-5, and G-5 on the left side. Thus, injections of the dilutions of the replications of each pair of tuberculins unfortunately were not placed on exactly corresponding sites on the opposite sides of the individual animals, as was done in experiment 1; however, they were placed in the same order on all animals.

Immediately after this experiment was carried out, some evidence was obtained that various skin areas of cattle differed in their ability to react to the intradermal injection of tuberculin. (This has been reported by Johnson,[10] Wadley,[16] Larsen *et al.*,[13] Baisden *et al.*[2] and, more recently,

TABLE 3—Comparison of Tuberculins by a Statistical Design of Placements of Injections in Four Cattle Sensitized to Mycobacterium Tuberculosis var. Bovis

	Av. for dilutions†	
Type*	1/25	1/250
Asparagine-1	2.19	1.30
Ammonium glutamate-1	2.64	1.55
Asparagine-2	2.77	1.32
Ammonium glutamate-2	2.59	1.69

* Tuberculins in pairs 1 and 2 of experiment 1. † Average of eight replications of each tuberculin per animal, given in millimeters of increase in skin thickness.

TABLE 4—Comparison of Tuberculins by a Statistical Design of Placements of Injections in Five Cattle Sensitized to Mycobacterium Tuberculosis var. Bovis

Type	Age of tuberculin at time of test* (mo.)	Av. for dilutions†	
		1/25	1/250
Asparagine-1	15	4.14	2.18
Asparagine-2	15	6.31	3.18
Asparagine-3	7	3.48	2.08
Asparagine-4	7	6.56	3.51
Ammonium glutamate-1	15	2.95	1.51
Ammonium glutamate-2	15	6.51	3.39
Ammonium glutamate-3	7	6.98	3.94
Ammonium glutamate-4	7	7.02	3.85
Asparagine average		5.12	2.73
Ammonium glutamate average		5.87	3.18

* The considerable variation between the materials of each type does not seem to be correlated with age.
† Averages of four replications of each tuberculin per animal, given in millimeters of increase in skin thickness.

by Larsen *et al.*[12]) The upper part of the side near the back was of low sensitivity, and the middle part and lower part of the sides were of medium sensitivity, while the middle and lower parts of the neck and the front flanks were of high sensitivity. Unfortunately, these areas are of unequal sizes, and the orderly placements of the injections made the individual tuberculins of a pair fall unequally on skin sites of the same sensitivity. Thus the effects of variations in sensitivity were confounded with the effects due to differences in potency of the tuberculins. Strict comparisons between the potencies of a pair of tuberculins could not be made. However, the tuberculins of each type were distributed equally over the three types of skin areas. Of the 15 replications of each type of tuberculin (5 asparagine tuberculins × 3 replications each, and the same for 5 ammonium glutamate tuberculins), nine replications fell on areas of medium sensitivity, three on low, and three on high. The injections were placed in numerical order, but were not properly paired.

While the conclusions reached from statistical studies of the data were not valid, the average results obtained on the 75 replications of the tuberculins of each type on all cattle of each sensitization appeared to be worthy of presentation (table 2).

Experiment 3.—The tuberculins making up pairs 1 and 2 of experiment 1 were retested at 25- and 250-fold dilutions on 4 cattle sensitized to *M. tuberculosis* var. *bovis*. By this time, differences in sensitivity of various skin areas and lack of differences between sides of an individual animal had been recognized; therefore, each of the four tuberculins was replicated on each of four general areas on each side of an animal. The areas were back, upper side, lower side, and neck and flank. This made eight replications per animal. The eight solutions (two dilutions of each of the four tuberculins) were placed at random in each area, those of the same tuberculin not always being adjacent.

By mistake, the neck area was not used exactly in the way intended, but this did not invalidate conclusions drawn from a study of the data, since the area was used in the same way on all animals and received all solutions. Averages of the reactions for the eight replications on each animal are given (table 3). These averages are low because of poor sensitization of the animals.

Experiment 4.—Two each of asparagine and ammonium glutamate tuberculins approximately 15 months old and two each approximately 7 months old were tested on 5 cattle sensitized to *M. tuberculosis* var. *bovis*. Each tuberculin was tested at 25- and 250-fold dilutions. Since it had been found that the two sides of an animal did not differ in sensitivity, corresponding areas on the two sides were combined, giving four areas large enough for 16 sites in each. The 16 solutions (4 asparagine and 4 glutamate tuberculins, each at 2 dilutions) were injected in each area on an animal at sites selected at random. Thus, there were four replications of each tuberculin per animal.

The results were studied by an analysis of variance.[16,17] The average reactions are shown (table 4).

SUMMARY

Formulas for two synthetic mediums used for years in the production of intradermic tuberculin for testing in the U.S. Department of Agriculture Tuberculosis Eradication Program are given. The first medium used L(−) asparagine as the source of nitrogen, while the second used ammonium L(+) glutamate. Fourteen tuberculins derived from the growth of *Mycobacterium tuberculosis* var. *hominis*, strains PN, DT, and C, on the first medium were compared for potency with 14 derived from the growth of these organisms under the same conditions on the second medium.

The comparison was made in four series of tests carried out on cattle artificially sensitized by intraperitoneal instillation of either heat-killed *Mycobacterium tuberculosis* var. *bovis* or heat-killed *Mycobacterium paratuberculosis*.

The results indicated that, in three of four series of tests, the tuberculins derived from the growth of the organisms on the ammonium glutamate medium were more potent than those derived from the growth on the asparagine medium.

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Stability of the Virus of Infectious Bovine Rhinotracheitis

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IN THE ORIGINAL report of Madin *et al.*,[3] on the isolation of the virus of infectious bovine rhinotracheitis (IBR), it was stated that the virus would survive at −70 C. for at least seven months and at 37 C. for at least 96 hours. Schwarz *et al.*[5] recently reported that the lyophilized IBR virus vaccine was stable, and no loss of titer could be demonstrated when it was held at 4 C. for eight months.

To facilitate handling of the virus and investigation of the disease in this laboratory, it has been necessary to determine more concerning the properties of this agent. This paper presents what has been learned relative to the influence of temperature, pH, lyophilization, and various chemicals on the infectivity of the virus.

MATERIALS AND METHODS

Bovine Kidney Tissue Culture.—The bovine kidney tissue cultures were prepared in this laboratory after the method of Madin *et al.*[3] The bovine kidneys were obtained from young adult cattle at a local slaughterhouse. The tissue culture medium was 0.5 per cent lactalbumin hydrolysate in modified Hank's balanced salt solution, containing 0.01 M tris-(hydroxymethyl aminoethane) adjusted to a pH of 7.4. Penicillin (500 units/ml.), streptomycin (500 μg./ml.), mycostatin (100 units/ml.), phenol red (0.002%), and 5 per cent lamb serum were incorporated in all mediums. One-milliliter and 50-ml. amounts of 0.5 per cent packed cells in medium were placed in 16- by 150-mm. tubes and 32-oz. prescription bottles, respectively. All cultures were incubated at 35 C. in a stationary position.

Virus.—The IBR virus was obtained* in tissue culture fluid from the fifteenth bovine kidney passage. Stock virus pools were prepared by inoculating 32-oz. prescription bottles of bovine kidney monolayer tissue culture with the virus and harvesting the infected tissue culture fluid at approximately 72 hours, when the cells were released from the glass surface. At this time, the pH of the tissue culture fluid was 7.0, and titration of the virus revealed a 50 per cent cytopathogenic end point (t.c.i.d_{50}) of $10^{-6.5}$ per 0.1 ml. After it was tested for bacterial sterility, the infected tissue culture fluid was dispensed into 2-ml. ampules, sealed, and stored in a mechanical deep freeze at −60 C. The growth and cytopathogenic effect of this virus in bovine kidney tissue culture was effectively and specifically neutralized by numerous convalescent serums from natural cases of IBR.

When the virus was administered intranasally to susceptible calves, a mild form of IBR was produced consistently. Intranuclear inclusions, like those described by Cheatham and Crandell,[1] in tissue cultures of bovine kidney and human amnion infected with IBR were demonstrated in the nasal mucosa of calves following intranasal inoculation.

Titration of Virus.—Tenfold serial dilutions of the virus were made in tissue culture mediums. One tenth milliliter of each dilution was inoculated into each of four bovine kidney tissue culture tubes. The inoculated tubes were observed for four days for cytopathogenic effect, and the t.c.i.d_{50} was calculated by the method of Reed and Muench.[4]

EXPERIMENTAL PROCEDURES AND RESULTS

The pH of Solutions.—Stock solutions of 0.1 M Na_2HPO_4 and KH_2PO_4 in distilled water were prepared and the two were combined to give the desired pH. One part of the stock virus was diluted in 9 parts of the buffer for each pH studied. The final pH of the virus-buffer mixtures, stored at 4 C., remained constant for the test periods. Samples were taken periodically and titrations made on bovine kidney tissue cultures.

The results obtained from exposing the virus of IBR to different pH levels for the periods indicated are shown (table 1). At a pH of 4.4, there was a 1-log decrease in tissue culture infectivity in two days and complete inactivation by the twentieth day. At a pH of 5.0, there was a 1-log loss at five days, and inactivation was complete by the twentieth day. When exposed to pH's of 6.0, 7.0, 8.0, 9.0, and 9.3, the original $10^{-6.5}$ t.c.i.d_{50} persisted for 25 days and there was a uniform loss of 2 logs on the sixtieth day, indicating a high degree of stability over this wide pH range. In view of these results, we continue to use diluents with a pH of 7.4, which is optimum for the tissue cultures used.

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TABLE 1—The Effect of pH on the Infectivity of Infectious Bovine Rhinotracheitis Virus*

pH	Time	Virus titer
4.4	1 day	6.5
	2 days	5.5
	5 days	4.5
	10 days	1.5
	20 days	Inactivated
5.0	2 days	6.5
	5 days	5.5
	10 days	4.5
	15 days	3.5
	20 days	Inactivated
6.0	25 days	6.5
	60 days	4.5
7.0	25 days	6.5
	60 days	4.5
8.0	25 days	6.5
	60 days	4.5
9.0	25 days	6.5
	60 days	4.5
9.3	25 days	6.5
	60 days	4.5

* The different pH's were maintained in 0.1 M phosphate buffers at 4 C.

Temperature.—During the studies of temperature stability, portions of the stock virus were held at temperatures of −60 C., 4, 22, 37, and 56 C. Samples were taken at the indicated intervals and titrations were made on bovine kidney tissue cultures.

The results of exposing the virus of IBR to different temperatures for varying periods are shown (table 2). After nine months' storage at −60 C., the titer was unchanged. At 4 C., there was no loss of titer after 30 days, but a 1-log loss occurred after 40 days. At 22 C., although there was no loss of infectivity after three days, a loss of 1 log was observed after five days and complete inactivation by the fiftieth day. When the virus was held at 37 C., a 1-log decrease in titer was evidenced by the third day, and inactivation was complete by the tenth day. When exposed to 56 C., the virus was inactivated in less than 21 minutes.

Lyophilization.—The stock virus was lyophilized by sublimation in a commercial mechanical freeze dryer. The vacuum was replaced with extra dry nitrogen before the ampules were sealed. Reconstitution and titration of the virus after drying revealed a 1-log loss of virus from the drying process. The titer remained unchanged after an additional 60 days at −60 C. When held at 35 C. for 30 days, the virus had a titer of $10^{-3.5}$.

EFFECT OF CHEMICALS

Ether.—Anesthetic ether was mixed with an equal amount of stock virus and shaken vigorously. After separation, the aqueous portion containing the virus was titrated in tissue culture. Virus so exposed to ether for one minute was inactivated, as demonstrated by the absence of cytopathogenic effect.

Acetone and Ethyl Alcohol.—Equal amounts of stock IBR virus were added to acetone (C.P.) and to ethyl alcohol (70% and 95%). When mixed with either of these reagents, the virus was inactivated in less than one minute, as evidenced by titration in tissue culture.

Formalin.—Formalin (formaldehyde solution, 40% U.S.P.) was added to the stock IBR virus to make final dilutions of formalin of 1:500, 1:1,000, and 1:5,000. While the virus-formalin mixtures were held at 4 C., samples were withdrawn at intervals for inoculation into tissue culture. Since, in control studies, a concentration of 1:10,000 of formalin was found toxic to tissue culture, a further 10^{-2} dilution of each virus-formalin suspension was used as inoculum for the bovine kidney tissue culture.

The virus was present in the 1:500 formalin dilution at the end of one hour, but not at the end of 24 hours. In the 1:1,000 formalin dilution, the virus was present at 24 hours, but was inactivated at 48 hours. In the 1:5,000 dilution, the virus was still active at 72 hours, but not at 96 hours.

TABLE 2—Effect of Different Temperatures on the Infectivity of Infectious Bovine Rhinotracheitis Virus

Temperature	Time	Virus titer
−60 C.	9 months	6.5
4 C.	30 days	6.5
	40 days	5.5
22 C.	3 days	6.5
	5 days	5.5
	19 days	4.5
	34 days	2.5
	42 days	0.5
	50 days	Inactivated
37 C.	1 day	6.5
	3 days	5.5
	5 days	3.5
	6 days	2.5
	10 days	Inactivated
56 C.	1 minute	6.5
	3 minutes	3.5
	12 minutes	2.5
	18 minutes	1.5
	21 minutes	Inactivated

SUMMARY

The influence of temperature, hydrogen ion concentrations, and selected chemical

agents upon the infectivity of the virus of infectious bovine rhinotracheitis were evaluated by titrations in bovine kidney tissue culture.

When suspended in tissue culture mediums at pH 7.0, the virus was found to be unusually stable, retaining its original titer for 30 days at 4 C. and losing only 1 log of infectivity after five days at 22 C.

Although loss of titer was rapid at pH's of 5.0 and 4.4 in 0.1 M phosphate buffer, the virus was remarkably stable over the pH range of 6.0 through 9.0. Only 2 logs of infectivity were lost after 60 days at 4 C., at pH's of 6.0, 7.0, 8.0, and 9.0.

Exposure of the virus to equal parts of ether, alcohol, or acetone caused prompt inactivation.

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Serological Studies with the Virus of Vesicular Stomatitis
I. Typing of Vesicular Stomatitis Viruses by Complement Fixation

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CUICA [2] first applied the complement-fixation test to foot-and-mouth disease while working with guinea pigs in 1929. Traub and Mohlmann [6] and Traub and Manso Rodriguez [5] discovered that convalescent guinea pig serums would react with bovine antigens, whereas previous workers using bovine serums had been unsuccessful.[8] Traub and Mohlmann were working at Reims, Germany, and their findings were not generally available until after World War II.

Since the first application of the complement-fixation test to foot-and-mouth disease, it has been widely accepted and is now used throughout the world by leading laboratories working with this disease, for typing vaccine strains, for identifying samples received from field cases, and for detecting variants. The rapidity and inexpensiveness of the complement-fixation test give it advantages over time-consuming and costly procedures requiring animal inoculation.

Our problem in the United States, where foot-and-mouth disease does not exist, is to make a rapid differential diagnosis of vesicular diseases. With foot-and-mouth disease foremost in mind, a test designed for that disease was adapted to include vesicular stomatitis. Work with vesicular stomatitis has been facilitated by the use of egg-propagated virus, which provides a potent and inexpensive antigen.[7]

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Vesicular stomatitis complement-fixation diagnostic work at Beltsville, Md., was started in September, 1950, by the authors. Some minor modifications in the test have been made during the last few years.

MATERIALS AND METHODS

Diluent.—Veronal buffer, as described by Brooksby,[1] is used as a diluent throughout the test.

Preparation of Ovine Erythrocytes.—Defibrinated blood, fresh or in Alsever's solution, from a normal sheep is centrifuged for ten minutes at 2,000 r.p.m. The packed cells are washed in at least ten times their volume of saline solution for three washings. The erythrocyte suspension is standardized by preparing a 3 per cent suspension of the washed packed cells. Additional accuracy in standardization is obtained by optical density measurements with the spectrophotometer as follows: 1 cc. of this suspension is lysed in 14 cc. of distilled water and 2 cc. is transferred to a cuvette (12 by 75 mm.) and is standardized against a water blank to an optical density (OD) of 0.34 ± 0.01 at a wave length of 550 mμ, using a Coleman junior spectrophotometer. Adjustments, when necessary, are made according to the following formula:

$$V_2 = \frac{V_1 OD_1}{0.34}$$

V_1 is the volume of suspension yielding OD_1, and V_2 the final volume at OD 0.34. (For example: if V_1 is 98.0 cc. and OD_1 is 0.37, then V_2 equals $\frac{98.0 \times 0.37}{0.34}$, or 106.6 cc.). Adjustment is made by adding 8.6 cc. of buffered saline solution and rechecking the OD of the adjusted suspension. If necessary, the diluent may be removed by centrifuging a portion and removing the required amount. Ovine blood preserved in Alsever's solution and stored at 4 C. may be used for six weeks. Cells for the day's tests are washed each morning.

Amboceptor Titration.—The amboceptor, or hemolysin, is produced in rabbits as described by Darter.[3] Titration of blood serum from the ear vein of each rabbit should be made daily, starting the third day after the last injection of ovine erythrocytes, and blood samples taken from the rabbits at their maximum titers. Hemolytic amboceptor is titrated by two rows of serial dilutions running from 1:100 and 1:150 stock dilutions to 1:6,400 and 1:9,600, respectively.

After the 1:100 and 1:150 stock amboceptor

TABLE 1—Complement Titration, Based on 5 Per Cent Stock Complement

	Complement percentages						
	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Complement (5%)	0.1*	0.15	0.2	0.25	0.3	0.35	0.4
Saline solution (compensate to 1 cc.)							
Saline solution	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sensitized cells	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total volume	2.5	2.5	2.5	2.5	2.5	2.5	2.5

* Expressed in cubic centimeters.
Incubate in water bath at 37 C. for 15 minutes and read immediately.

dilutions are made, 1 cc. is carried over to the lowest dilution in its row and twofold dilutions are made, progressing to the end of the row by carrying over 1 cc. to the next tube and discarding 1 cc. at the end of the row. One cubic centimeter of 5 per cent complement (excess complement) is added to each tube, followed by saline solution and standardized ovine erythrocytes. These are thoroughly mixed by shaking, incubated in a water bath at 37 C. for 20 minutes, then read immediately.

The amboceptor unit is the highest titration dilution producing complete hemolysis. Amboceptor is filtered, dispensed in tubes, inactivated at 56 C. for 30 minutes, phenolized as described for serum, and stored in the refrigerator at 4 C.

Sensitized Cells.—The sensitized cells are prepared as follows: (1) Amboceptor dilution (100 cc.) is prepared in veronal buffer to contain a final concentration of 2 units of amboceptor, as used in the test. (For example, an amboceptor titrating 1:4,000 would be used as 1:2,000, or 0.05 cc. of undiluted amboceptor plus 99.95 cc. of saline solution.) (2) A standardized ovine erythrocyte suspension (100 cc.) in veronal buffer is prepared.

Ingredients 1 and 2 are prepared separately, and then the amboceptor solution is added to the cell suspension with immediate shaking for equal absorption of the antibody on the cells. This mixture is placed in the 37-C. water bath for ten minutes for sensitizing. The sensitized cells are then held in the refrigerator and used whenever needed during that day. They are placed at room temperature about ten minutes before use.

Complement.—Complement is prepared from normal, healthy, adult guinea pigs which are fasted for 24 hours before the taking of blood samples. Groups of 10 or more guinea pigs are exsanguinated, and the blood is allowed to clot at room temperature. The serums can be individually titrated for complement activity. Those serums showing satisfactory activity are pooled, sealed in ampules, quick-frozen in a bath of dry ice and alcohol, and stored at −70 C.

Complement Titration.—The complement titration, based on 5 per cent stock complement, is outlined (table 1). A unit of complement is the smallest amount that produces sparkling hemolysis, and the percentage used in the test is 1.6 times 1 unit (3% unit × 1.6 = 4.8%). Quantities used in complement titrations in the presence of antigen are halved to the regular test volume in order to conserve antigen. The 0.5 per cent complement dilutions are omitted, and the dilutions include 2.0 to 7.0 per cent complement.

Antigens.—The antigen is prepared from vesicular material of infected horses, cattle, sheep, swine, or guinea pigs by grinding in a mortar, using sterile ground glass as an abrasive. Phosphate buffered saline solution is added to make a 20 per cent suspension, which is centrifuged twice at

TABLE 2—Method for Antigen Titration

Tube No.	Saline solution (cc.)	Antigen preparation of dilutions	Final antigen dilution	Serum (cc.)	Complement (cc.)	Sensitized cells (cc.)
1	.....	0.25 cc. undiluted antigen	Undiluted	0.25†	0.25	0.5
2	0.25	0.25 cc. undiluted antigen	1:2*	0.25	0.25	0.5
3	0.25	0.25 cc. from tube 2	1:4	0.25	0.25	0.5
4	0.25	0.25 cc. from tube 3	1:8	0.25	0.25	0.5
5	0.25	0.25 cc. from tube 4	1:16	0.25	0.25	0.5
6	0.25	0.25 cc. from tube 5	1:32	0.25	0.25	0.5
7	0.25	0.25 cc. from tube 6	1:64	0.25	0.25	0.5
8	.....	Specificity test 0.25 cc. undiluted antigen.	Undiluted	0.25**	0.25	0.5
9	.....	Specificity test 0.25 cc. undiluted antigen.	Undiluted	0.25	0.25	0.5

* Antigen and saline solution are mixed and carried over in tubes 2 through 7 only, the 0.25 cc. surplus from tube 7 being discarded; † a homologous guinea pig serum dilution of 1:5 or weaker is used to titrate the antigen; ** a known specific heterologous hyperimmune guinea pig serum of 1:5 dilution and a normal guinea pig serum of 1:5 dilution are added to the specificity test to determine the purity of antigens, as specificity tests must not show nonspecific fixation.

Saline solution, antigen, serum, and complement should be shaken and incubated in a water bath at 37 C. for 20 minutes. After the sensitized cells are added to the test, the tubes are shaken and incubated for an additional 30 minutes at 37 C.

TABLE 3—Controls to Accompany Each Test

Purpose of control	Label on tube	Antigen (cc.)	Serum (cc.)	Saline solution (cc.)	Complement (cc.)	Sensitized cells (cc.)
Serum—anticomplementary	SC 1	.....	0.25	0.25	0.25	0.5
Serum—hemolytic	SC 2	.....	0.25	0.5	.....	0.5
Antigen—anticomplementary	AC 1	0.25	.....	0.25	0.25	0.5
Antigen—hemolytic	AC 2	0.25	.....	0.5	.....	0.5
Complement—1 dose *	0.25	.....	.....	0.5	0.25	0.5
Complement—½ dose	0.125	.....	.....	0.625	0.125	0.5
Complement—¼ dose	0.06	.....	.....	0.69	0.06	0.5
Sensitized cells	HS	.....	.....	0.75	.....	0.5

* One dose is the amount of complement that is used throughout the test.

Antigen, saline solution, serum, and complement are shaken and incubated in a water bath at 37 C. for 20 minutes. After the sensitized cells are added to the test, the tubes are shaken and incubated for an additional 30 minutes at 37 C.

2,500 r.p.m. or faster for 20 minutes and used undiluted, without inactivation. Vesicular lymph provides a high-titer antigen but it is seldom available for this purpose. Only two immunologically different types of vesicular stomatitis virus (New Jersey and Indiana) are known to exist.

Antigen Titrations.—The method for antigen titrations is outlined (table 2). Serum controls to detect anticomplementary or hemolytic serums, and antigen controls to detect anticomplementary or hemolytic antigens, are included for each serum and antigen used in the test (table 3).

When a test containing New Jersey- or Indiana-type antigen, to be tested with serums of both types, is being set up, if an antigen of the heterologous type is not being tested at the same time, it is preferable to include one as a known test-control antigen. This assures the serologist that the heterologous system is functioning. Similarly, a known heterologous serum is included when unknown serums are being tested.

If there is a possibility that all the unknown serums being tested are negative, leaving no proof that the complete complement-fixing system is working, a known serum or antigen of each type is added.

Preparation of Hyperimmune Guinea Pig Serum.—Convalescent guinea pigs, whose footpads have sufficiently healed from the primary inoculation of a guinea pig passage of vesicular stomatitis virus 20 to 40 days previously, are hyperimmunized by one to three hyperimmunizing doses at three- to four-day intervals. Material for hyperimmunization is a 10 per cent suspension of virus-infected guinea pig epithelium, prepared in saline solution buffered at pH 7.6. After centrifuging at 2,000 r.p.m. for five to ten minutes, the inoculum is injected intracutaneously into the metatarsal pads, using 0.25 cc. per pad. Streptomyin (2.5 mg./cc.) and penicillin (5,000 units) may be added to control bacterial contamination. One week after a single dose, or the second day after repeated doses, the hyperimmunized guinea pigs are exsanguinated. The serums are collected and tested individually for titer and specificity. For hyperimmunizing, guinea pig passages are preferably kept to a maximum of not more than five passages from the field sample to prevent modification of the virus.[6] A high proportion of type-specific serums is obtained.

Serum Titrations.—Each hyperimmune guinea pig serum is titrated and tested for type specificity. Bovine antigens rather than egg antigens are used to check specificity, as bovine antigens more closely

TABLE 4—Serum Titration as Used for a New Jersey Type Serum

Tube No.	Purpose	Saline solution (cc.)	Serum 1:5 (cc.)	Preparation of dilution	Serum dilution	Antigen (cc.)	Antigen type	Complement (cc.)	Sensitized cells (cc.)
1	Titration	.....	0.25	1:5 serum	1:5	0.25	N.J.	0.25	0.5
2	Titration	0.25	0.25	Equal parts 1:5 serum and saline solution	1:10	0.25	N.J.	0.25	0.5
3	Titration	0.25	.....	0.25 cc. from tube 2	1:20	0.25	N.J.	0.25	0.5
4	Titration	0.25	.....	0.25 cc. from tube 3	1:40	0.25	N.J.	0.25	0.5
5	Titration	0.25	.....	0.25 cc. from tube 4	1:80	0.25	N.J.	0.25	0.5
6	Titration	0.25	.....	0.25 cc. from tube 5	1:160	0.25	N.J.	0.25	0.5
7	Titration	0.25	.....	0.25 cc. from tube 6*	1:320	0.25	N.J.	0.25	0.5
8	Specificity	.....	0.25	1:5 serum	1:5	0.25	Ind.	0.25	0.5
9	Specificity	.....	0.25	1:5 serum	1:5	0.25	Normal	0.25	0.5
10	Double serum	.....	0.5	1:5 serum	1:5	.....	.....	0.25	0.5

* Mix serum and saline solution in tubes and carry over in tubes 2 through 7 only, discarding the 0.25-cc. surplus from tube 7.

For Indiana type serums, the Indiana antigen would be used in the titration and New Jersey antigen in the specificity. The double serum control (tube 10) is sometimes useful in detecting slightly anti-complementary serums, and its use is optional.

Saline solution, serum, antigen, and complement are shaken and incubated in a water bath at 37 C. for 20 minutes. After the sensitized cells are added to the test, the tubes are shaken and incubated for an additional 30 minutes at 37 C.

resemble the field sample viruses which will be tested. The titration dilutions are made similarly to those in the antigen titration, except that a 1:5 serum dilution is used to start the dilution series and is used in specificity tests and serum-anticomplementary and hemolytic controls. All guinea pig serums are inactivated at 56 C. for 30 minutes and tested as outlined (table 4).

Ten per cent bovine antigens are prepared and titrated against known serums. The heterologous antigen is used at 10 per cent to detect nonspecific serums. The homologous antigen is used at 5 per cent to determine the relative titers of the serums. The percentage of antigens used may vary according to the antigen titration.

Two serum controls for each serum are included in all serum titrations or specificity tests. The serum control with complement, and with saline solution replacing the antigen, detects any anti-complementary effect of the serum. This tube must show complete hemolysis. The serum control without complement or antigen is added to detect improper inactivation of the serum and other causes of hemolysis which may occur. This tube must be free of hemolysis.

laboratory in 50 per cent glycerine phosphate buffer at pH 7.6. These vesicle covers may be taken from the tongue, foot, snout, or udder of an infected animal.

The antigen is prepared by washing about 2 Gm. of the material once or twice with saline solution to remove the bulk of the glycerine. The tissue is finely ground with a mortar and pestle, and saline solution is added to make a 20 per cent antigen suspension. The material is centrifuged at 2,500 r.p.m. for 20 minutes, transferred to a second tube, and recentrifuged at 2,500 r.p.m. for 20 minutes. If only a small sample is received, a 10 or 15 per cent antigen suspension is prepared. The centrifuged supernatant antigen is tested without further dilution.

How a field antigen is tested against a series of known vesicular stomatitis and foot-and-mouth disease hyperimmune antiserums is shown (table 5). Anti-vesicular

TABLE 5—Scheme for Field Sample Typing

Tube No.	1	2	3	4	5	6	7	8
	Guinea pig serums (cc.)						Antigen control (cc.)	
	N.J.	Ind.	A	O	C	Normal	AC 1	AC 2
Unknown antigen undiluted	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Serum (type indicated in column headings)	0.25	0.25	0.25	0.25	0.25	0.25	.....	.....
Saline solution	.....	.....	.....	.....	.....	.....	0.25	0.5
Complement	0.25	0.25	0.25	0.25	0.25	0.25	0.25	.....
Sensitized cells	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Additional types of anti-foot-and-mouth disease serums may be added in vertical rows following A, O, and C.

Antigen, serum, saline solution, and complement are shaken and incubated in a water bath at 37 C. for 20 minutes. After the sensitized cells are added to the test, the tubes are shaken and incubated for an additional 30 minutes at 37 C.

After the serums are tested, they are pooled according to titer and specificity. The high-titer, specific serums are reserved for typing of field samples.

Pools of serum are filtered through Berkefeld N filters or Mandler No. 7 to 11, placed in sterile tubes, inactivated at 56 C. for 30 minutes, phenolized to 0.5 per cent (9 parts of serum filtrate plus 1 part of 5% phenol), and stored under refrigeration at 4 C. Each pool is tested for titer, specificity, and anticomplementary effect. It is important for type differentiation of field samples to use serums which show no anticomplementary action, as they will increase the anticomplementary effect of poor field samples.

TYPING OF FIELD SAMPLES

For type differentiation of virus samples from the field, covers of vesicles, preferably unruptured, should be sent to the

stomatitis serums are usually diluted 1:5, while anti-foot-and-mouth disease serums are diluted 1:16. Vesicular stomatitis serums may be used 1:5 without cross-type reactions. The dilution of foot-and-mouth disease serums varies according to their titer, and they are used in a higher dilution to reduce cross-type reactions. Normal serum dilution 1:5 is also included. Test controls are outlined in table 3. The complement unit is determined by titrating complement in the presence of antigen. Each antigen is tested, using 1.6 times the complement unit, as described.

An alternate method of typing an unknown field virus may be used without titrating complement in the presence of the unknown antigen. The alternate method uses four identical sets of each field

TABLE 6—Summary of Known Antigens from Experimental Animals

Complement-fixation reaction	N.J. vesicular stomatitis			Ind. vesicular stomatitis			Normal	
	Bovine	Porcine	Equine	Bovine	Porcine	Equine	Bovine	Porcine
Positive N.J. vs	24	13	8	0	0	0	0	0
Positive Ind. vs	0	0	0	24	9	7	0	0
Positive FMD (A, O, C)	0	0	0	0	0	0	0	0
Negative vs	0	0	0	0	0	0	6	4
Total (95)	24	13	8	24	9	7	6	4

antigen with serum. The complement dose is increased 1.0 per cent, with each set starting 0.5 per cent above one unit. For example, with a complement unit of 2.5 per cent determined in the presence of saline solution (table 1), a field antigen would be tested using 3.0, 4.0, 5.0, and 6.0 per cent complement. If the antigen sample is insufficient, only 3.0 and 5.0 per cent would be tested or, occasionally, with only sufficient virus for one series, 4.0 per cent would be selected. The tissue composing the virus sample, the age of the vesicle at harvest, bacterial contamination, method of preservation, and the percentage of antigen concentration are factors which may increase the anticomplementary activity.

Considering these irregularities in the quality of a virus sample received from the field and the occasional testing of a sample in which the anticomplementary effect is not overcome by the highest of the four complement percentages, the complement titration in the presence of the unknown antigen is to be preferred. The antigen, serum, and complement are incubated at 37 C. for 20 minutes in a water bath. The indicator system is then added and incubated at 37 C. for 30 minutes.

A preliminary reading may be made when the tubes are removed from the water bath, but greater accuracy for the final reading is obtained following centrifugation or by allowing the completed test to settle in the refrigerator overnight and making the reading the following day. Negative samples show complete hemolysis. A 4+ reaction shows no hemolysis and represents a total fixation of the complement.

Complement-fixation results of 95 known antigens from experimentally infected animals and 170 samples received from the field have been summarized (tables 6, 7). Ten antigens made from normal epithelium are included.

Type-specific results were obtained in all work using antigens made from vesicle covers of animals with vesicular stomatitis.

This test has not been found applicable to vesicular exanthema, partially because of the lack of a good hyperimmune guinea pig serum. Attempts to propagate vesicular exanthema virus in animals have failed to provide a satisfactory source of virus or immune serum other than from swine. As

TABLE 7—Summary of Field Antigens Tested

Complement-fixation reaction	Suspected vesicular stomatitis			Vesicular exanthema, porcine*
	Bovine	Porcine	Equine	
Positive N.J. vs	51	33	11	0
Positive Ind. vs	3	1	0	0
Positive FMD (A, O, C)	0	0	0	0
Suspicious N.J. vs	2	2	0	0
Negative vs	29	6	0	32
Total (170)	85	42	11	32

* Diagnosed by animal inoculation.

other animals, such as guinea pigs, become sensitized to swine tissue by injections of the vesicular exanthema inoculum containing the tissue, no specific antibody appears available except in swine serum. Additional factors include the procomplementary activity of hog serum and the longer period of fixation required for hog serum.

DISCUSSION

The complement-fixation test for foot-and-mouth disease has been valuable in frequent typing of virus strains used for vaccine production. At one institution where several types of virus were being produced, accidental, unobserved mixing of the viruses resulted in the blocking out of the weaker virus, and vaccine production continued for some time before the type change was detected.[4]

As involved tests on immune animals are necessary to determine immunological types of the vesicular viruses, the complement-fixation test has become a rapid, inexpensive method for routine determination of virus type. Most countries working with foot-and-mouth disease are interested in testing virus samples from field cases, so

that they may control the disease by the use of homologous vaccines.

Field samples present a problem because of their frequent anticomplementary quality. Epithelium from ruptured vesicles rapidly decreases in titer and becomes anticomplementary. Preservation of virus epithelium in 50 per cent glycerine with 1/15 M phosphate buffer solution at pH 7.6 is preferred.

The complement-fixation test for vesicular stomatitis and foot-and-mouth disease differs from that for many bacterial diseases and for vesicular exanthema, in that the antigens are capable of fixing large quantities of complement.

SUMMARY

A simple complement-fixation test which is reliable for the differential diagnosis and typing of vesicular stomatitis and foot-and-mouth disease is described in detail. Epithelial coverings removed from the vesicles of infected animals in field epizooties are tested with serums of guinea pigs hyperimmunized with each of the virus types.

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The Quantitative Determination of Mercury in Animal Tissues

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CERTAIN mercurial preparations are used for the treatment and prevention of fungus infection in cereal grain and, in this way, are not uncommon sources of poisoning in animals. Since many mercurial salts are volatile, any digestion technique requiring heat used in testing the mercurial content of tissues may cause significant loss of the metal. In the method reported here, this source of error is overcome by the relatively low temperature digestion permitted by the use of a combined condenser-cold finger apparatus.

MATERIALS AND METHODS

Apparatus.—The apparatus (fig. 1) consisted of a Pyrex water-cooled condenser sealed at one end to form a vessel with a volume of 10 ml. The top of the condenser had a standard taper 24/40 joint to accommodate a cold finger which extended to just above the bottom of the water jacket. A 2-mm. hole between the standard taper joint and the top of the water jacket acted as a vent for gas generated during digestion and as an opening for the addition of the oxidizing (permanganate) solution.

Reagents.—The reagents used were reagent-grade sulfuric acid (concentrated), reagent-grade potassium permanganate (saturated solution), hydroxylamine hydrochloride (5% solution), approximately 0.25 N hydrochloric acid (21 ml. concentrated/liter of solution), potassium bromide (40% solution), and fractionated chloroform (59.5–60.5 C. fraction collected).

Buffer.—The buffer consisted of 150 Gm. of disodium phosphate dodecahydrate and 38 Gm. of anhydrous potassium carbonate in 1 liter of solution. The solution was purified by extracting with dithizone and washing with chloroform, and was stored in a polyethylene or Pyrex bottle.

Dithizone Solution A (Stock).—This solution consisted of 100 mg. of pure dithizone dissolved in 800 ml. of redistilled chloroform. Dithizone solution B (weak) is prepared by diluting 50 ml. of solution A to 500 ml. with chloroform. Weaker solutions may be made by further dilutions if required.

Stock Solution of Mercury.—Mercuric chloride (6.767 Gm.) was dissolved in water and diluted to 1 liter. The standard solution was prepared by diluting 10 ml. of the stock to 1 liter with water.

Procedure.—The tissue to be analyzed was macerated in a blendor and a 1-Gm. aliquot was passed through the standard taper joint at the top of the condenser into the digestion vessel. Contamination of the inner sides of the apparatus was prevented by weighing the tissue into a gelatin capsule before passing it into the vessel. Connections were joined between the cold water line outlet and the condenser-cold finger inlets. Water (2 ml.) and sulfuric acid (3 ml.) were added to the tissue in the vessel and the cold finger fitted into the condenser.

The apparatus was placed on a water bath, with the digestion vessel submerged in the boiling water and heated with frequent agitation for 45 minutes after the tissue has been liquefied. Potassium permanganate solution (1 ml.) was added through the vent and mixed with the digest by agitation. When the foaming had stopped, another milliliter of permanganate was added and the contents were heated until no further color change was evident. Increments of potassium permanganate were added, with heating, until the contents became clear and had a light amber color.

The apparatus was taken from the water bath and the contents cooled under running water. The cold finger was raised and rinsed with 5 ml. of hydroxylamine hydrochloride solution, followed by distilled water. The digest was transferred from the condenser to a 250-ml. separatory funnel, the condenser rinsed several times with distilled water, and the rinsings added to the separatory funnel. The cold finger and condenser were rinsed with dithizone solution B and the rinsings added to the separatory funnel. The final volume of dithizone should be about 50 ml.

The funnel was shaken for two minutes, the layers allowed to separate, and the dithizone layer transferred to a second separatory funnel. The aqueous phase was washed with three 5-ml. portions of redistilled chloroform and the washings were transferred to the second separatory funnel. The dithizone layer was washed with 25 ml. of 0.25 N HCl and transferred to a third funnel. The HCl was washed with three 5-ml. portions of chloroform and the washings added to the third separatory funnel. The HCl solution was discarded. Fifty milliliters of 0.25 N HCl and 5 ml. of potassium bromide solution were added to the dithizone, and the solutions were shaken to transfer the mercury from the organic to the aqueous phase.

The dithizone layer was discarded and the aqueous layer washed with three 5-ml. portions of chloroform. The washings were discarded. The aqueous phase was adjusted to approximately pH 6 by the addition of 11 ml. of buffer and titrated with dithizone solution B. This was carried out by adding the solution in increments of 0.5 to 1.0 ml. and shaking after each addition.

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TABLE 1—Recovery of Mercury from Liver Tissue

Mercuric chloride added (μg./Gm.)	Recovery (%)				Average recovery (%)
0.0	0	0	0	0	0.0
5.0	94	94	94	110	98.0
12.5	96	96	100	100	98.0
25.0	96	96	100	98	97.5
50.0	96	98	102	100	99.0

If mercury is present, the organic phase takes on the salmon-orange color of mercury dithizonate. Any other color change indicates the end point. Estimation also may be made by the mixed color method.

The dithizone solution was standardized by titration with an aliquot of a standard solution of mercury in 50 ml. of 0.25 N HCl, 5 ml. of potassium bromide, and 11 ml. of buffer.

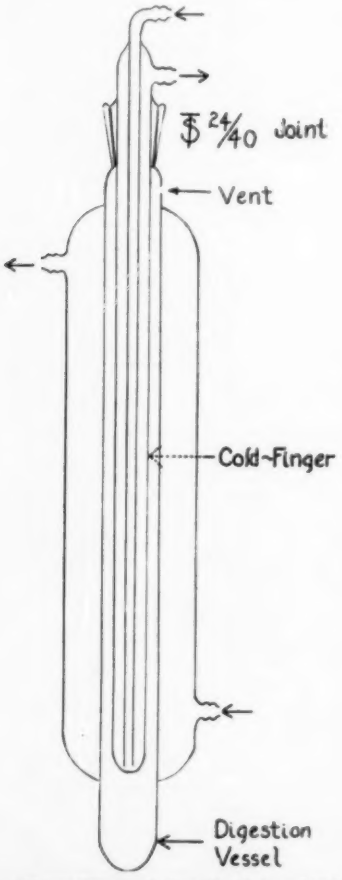

Fig. 1—Apparatus used for the determination of mercury in tissues.

The amount of mercury in the unknown is calculated by: The number of milliliters required for titration, multiplied by the value of dithizone solution, is equal to the micrograms of mercury per gram.

RESULTS

The results (table 1) were obtained by adding mercuric chloride to 1 Gm. of liver and then treating by the method described. The method also has been used successfully with brain, muscle, and kidney tissue.

DISCUSSION

The digestion flask is designed to have a minimum glass area [2] and a continuity of surface. The use of a similar cold finger adapted by a standard taper joint to a 50-ml. digestion flask resulted in an average recovery in three trials of only 55 per cent of the mercury added.

After digestion, the excess permanganate is reduced with hydroxylamine and the mercury is transferred from the acid aqueous to the organic phase as mercury dithizonate. Since silver dithizonate is unstable in the presence of dilute halogen,[1] the silver is separated from the mercury by washing the dithizone with dilute hydrochloric acid solution. The organic layer is shaken with an acid solution of potassium bromide, which destroys the mercury dithizonate, and the mercury passes to the aqueous phase as potassium mercuric bromide, leaving the copper in the chloroform layer.[1] Under the conditions described, (*i.e.*, extraction with dilute dithizone solution), bismuth will not interfere even if present at 100 times the concentration of mercury.[1] Finally, the solution is adjusted to pH 6 to permit the mercury to react with dithizone for estimation as mercury dithizonate. Gold and palladium may interfere, but neither is a common cause of poisoning in domestic or wild animals.

SUMMARY

A method is described for the recovery of mercury from and the quantitative determination of mercury in tissue. An average of 98 per cent recovery has been effected, within the range of 0 to 50 μg.

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Paper Electrophoretic Analysis of Rabbit Serum as an Aid in the Selection of Experimental Rabbits

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AMONG THE REPORTS that have appeared on the fractionation of the serum proteins of domestic animals, using the paper electrophoretic technique, are those of Chopard [4] and Boguth,[1,2] who observed the serum proteins of many domestic animals. Nilsson and Åberg [5] studied the normal cow, and Rooney [6] compared normal and abnormal bovine serum proteins. However, only Chiarioni and Nardi [3] and Chopard [4] appear to have used this method for the examination of the serum proteins of rabbits. The former workers compared paper and moving boundary electrophoretic patterns of normal rabbit serum. The latter briefly mentioned values in the rabbit but separated only four fractions. There was a wide range of values between the two reports and no mention of sex or breed was made.

The purpose of this work was to examine the serum protein patterns of normal, healthy rabbits, to establish normal ranges, and to observe possible breed and sex differences, using the paper electrophoretic method. During the course of the investigation, the value of this method as an aid in the selection of normal animals for experimental investigations became apparent.

MATERIALS AND METHODS

Thirty adult rabbits, equally divided as to sex and of various breeds, were obtained locally. The rabbits were isolated for two weeks in individual cages for observation, acclimation, and determination of pregnancy status. Parasitological examinations also were performed.

Blood samples were taken by cardiac puncture at weekly intervals for four weeks, and serum protein determinations were made by paper electrophoresis. Hanging strip paper electrophoresis was done, using a Durrum-type cell. The buffer system was sodium-barbital-barbituric acid, with an ionic strength of 0.075 and a pH of 8.6. Undiluted serum (0.008 ml.) was applied to Whatman 3-mm. filter paper strips. The separations were carried out for 16 hours at a constant current of 5 ma., eight strips being run at one time. The strips were dried and stained with bromphenol blue. After a 12-hour color equilibration period, the strips were analyzed by a Servo-type integration scanner.*

TABLE 1—Serum Protein Values of Abnormal Rabbits

Necropsy findings	No. animals	Mean percentage of total serum protein					No. samples per animal
		Albumin	*Alpha* 1	*Alpha* 2	*Beta*	*Gamma*	
Normal	18	64.01	7.21	5.80	12.77	9.91	3–4
Nephritis (interstitial)	4	48.40	8.77	6.75	17.50	18.10	3
Nephritis and acute lymphadenitis (interstitial)	1	35.35	19.91	19.91	19.67	25.77	3
Bile duct infected with *Eimeria stiedae*	1	40.90	7.75	6.97	13.98	30.00	3
Liver—minimal portal round cell infiltration	1	61.00	7.10	4.00	10.78	16.72	3
Liver—minimal portal round cell infiltration	1	60.25	5.47	5.28	11.75	17.20	3
Liver—hemorrhage and necrosis, and cecum—necrotic cecitis with lymphoid proliferation	1	47.66	9.18	8.59	15.73	18.90	3
Endocarditis (chronic)	1	22.80	31.60	12.53	21.70	11.33	3
Uterine cyst (left horn); 195 cc. proteinaceous fluid removed	1	45.75	11.67	10.10	12.50	19.40	2

RESULTS

The following normal serum protein percentages were obtained from 12 male and

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Taken from a thesis to be submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree.

* Analytrol model R scanner is manufactured by Specialized Instruments Corp., Belmont, Calif.

6 female rabbits: albumin, 64.01 ± 1.63 per cent; *alpha* 1 globulin, 7.21 ± 0.96 per cent; *alpha* 2 globulin, 5.80 ± 1.05 per cent; *beta* globulin, 12.77 ± 1.20 per cent; and *gamma* globulin, 9.91 ± 1.10 per cent. They are the average of three to four blood samples from each rabbit. These values were averaged and the mean value of each fraction was determined and expressed as the percentage of total protein (table 1). The values for the abnormal rabbits (table 1) were computed similarly. Representative scans of normal and abnormal rabbits are shown (fig. 1). An investigation of an additional 14 rabbits with *Eimeria stiedae* infection (table 2) were based on one blood sample per animal.

Migration values of normal animals are shown (table 3). Since the migration is constant within each run, but not from run to run, a C/AF value was used to allow comparison of migration values. The equation used cancels inherent variables between runs, such as different batches of buffer and line current fluctuations.

$$C/AF = \frac{\text{Distance of component migration (center of band) from origin}}{\text{Distance of albumin front from origin}}$$

These C/AF values were calculated for those components migrating toward the positive pole. *Gamma* globulin migration was calculated directly in a direction toward the negative pole.

TABLE 2—Serum Protein Values for Young Adult Rabbits with Eimeria Stiedae Infections

	Percentage of total serum protein				
Sex	Albumin	*Alpha* 1	*Alpha* 2	*Beta*	*Gamma*
F	45.60	8.20	5.85	15.75	24.60
F	43.40	6.28	4.40	13.20	32.60
F	35.90	6.12	5.31	17.50	35.00
F	45.00	9.75	7.50	18.00	19.50
F	49.50	7.32	6.50	17.05	19.55
F	44.00	8.68	5.80	15.22	26.10
F	37.20	9.30	5.59	19.50	28.30
F	42.00	8.56	6.44	15.00	27.80
M	53.50	9.36	4.92	11.70	25.60
M	53.80	8.02	5.05	8.78	24.00
M	44.70	8.70	4.95	14.25	27.30
M	53.00	7.80	5.60	11.20	22.40
M	41.00	7.05	3.84	17.30	38.20
M	30.20	10.80	5.88	14.30	38.60

DISCUSSION

In the normal group, there was greater variation among rabbits than between consecutive samples from a single rabbit. Reproducibility was determined by using the same serum on different runs. A range of 2 to 3 per cent between different analyses was found.

Breed and sex appeared to have no influence on the serum patterns or on the migration values. Insufficient numbers of animals were used to determine this statistically for breed differences.

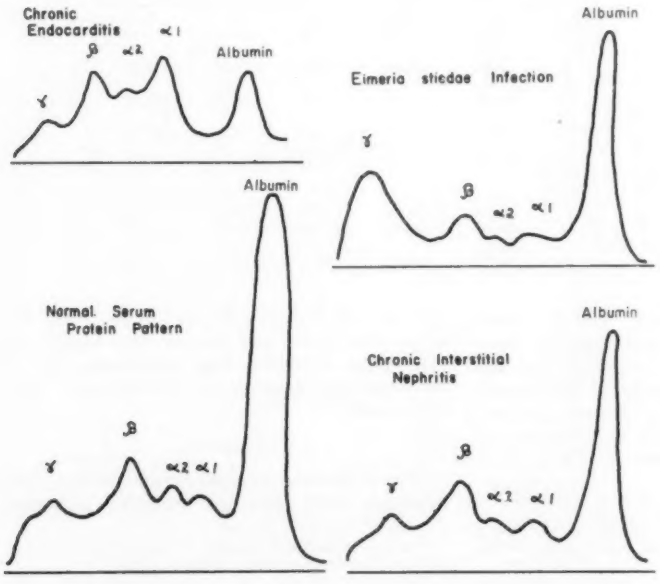

Fig 1—Normal and abnormal serum protein patterns of rabbits.

TABLE 3—Serum Protein Migration Values for Normal Rabbits (Expressed as C/AF Ratio*)

Sex	No.	C/AF ratios of serum protein components				
		Albumin	*Alpha* 1	*Alpha* 2	*Beta*	*Gamma* †
F	6	0.906 ± 0.001	0.596 ± 0.004	0.467 ± 0.009	0.328 ± 0.006	−501 ± 0.006
M	12	0.907 ± 0.006	0.609 ± 0.010	0.478 ± 0.009	0.334 ± 0.004	−501 ± 0.005
Both		0.906 ± 0.004	0.604 ± 0.008	0.474 ± 0.009	0.332 ± 0.005	−501 ± 0.005

* C/AF ratio = $\frac{\text{Distance of component migration (center of band) from origin}}{\text{Distance of albumin front from origin}}$

† Measured directly from origin to center of component band.

Chiarioni and Nardi[3] reported the following results for paper electrophoresis of rabbit serum: albumin, 57.21 per cent; *alpha* 1 globulin, 6.04 per cent; *alpha* 2 globulin, 5.72 per cent; globulin, 11.25 per cent; and *gamma* 1 and *gamma* 2, 16.96 per cent. In our study, none of the rabbits considered normal had an albumin level as low or a *gamma* globulin level as high as reported by these authors. Further, in our study, *gamma* globulin has been reported as a single fraction. With these exceptions, our results agree with those of Chiarioni and Nardi. They agree closely with the results given by Chopard,[4] who found 62.5 per cent albumin, 10.7 per cent *alpha*, 14.8 per cent *beta*, and 12 per cent *gamma* globulin.

Boundary electrophoretic values for the serum components of rabbits[7] agree within 1 or 2 per cent of the results obtained here, although a *theta* fraction was not apparent using paper, and in this work may be represented in the total *gamma* globulin.

Abnormal Rabbits. The 5 animals with kidney lesions (table 1) showed a marked decrease in albumin due to loss of albumin in the urine (as demonstrated by the Robert test) together with high *beta* and *gamma* globulin. The high *beta* and *gamma* globulin probably represent a change in ratio, rather than increased globulin production. The rise of *alpha* 1 and *alpha* 2 globulin appeared related to the amount of tissue inflammation and destruction present.

The animals with minimal portal round cell infiltration in the liver showed low normal albumin levels with a marked increase in *gamma* globulin. The other globulins from those animals considered normal showed little or no change. Whether the high *gamma* globulin level in these animals was the result of response to prior infection with resulting portal round cell infiltration is not clear.[8]

The remaining animals with more than one pathological change showed markedly abnormal serum patterns, but no attempt was made to correlate the patterns with any one specific lesion.

The rabbits (table 2) were young adults with a diagnosis of coccidiosis established by fecal examination. These animals were treated with sulfaquinoxiline for one month, and the values presented were obtained after the cessation of clinical signs. The findings were consistent with the varying degrees of liver damage found at necropsy.

SUMMARY

Paper electrophoretic patterns of 15 male and 15 female rabbits were studied It was determined that the serum fraction percentages of normal rabbits showed little variation, with no differences between sexes or breeds. The various abnormalities were indicated first by the serum patterns and later verified by necropsy findings.

In addition, 14 rabbits with clinically established cases of coccidiosis caused by *Eimeria stiedae* were investigated, using this method. These rabbits showed patterns consistent with varying degrees of liver damage.

The use of paper electrophoresis as an aid in the selection of normal animals for experimental investigations was brought out.

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The Influence of Antimetabolites on the Growth of Newcastle Disease Virus in Chorioallantoic Membranes Suspended in Tyrode's Solution

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THE VIRUS-INFECTED HOST is a complex biological unit in which biosynthesis of the virus is associated with the metabolism of the host. The influence of several compounds demonstrated to have some biochemical activity on the metabolism of the virus-host complexes *in vitro* was investigated, using Newcastle disease virus. These compounds were chosen because of their specific activity on enzymes that influence the formation of certain cellular components believed to be involved in virus synthesis.

MATERIALS AND METHODS

Virus.—The strain of Newcastle disease virus (NDV) selected for this study was the NJ-Roakin 1946 isolate.*

Virus Titrations.—The number of virus particles was determined by titrating the lethal activity of the virus for chicken embryos. Decimal dilutions of virus were prepared in nutrient broth. One tenth milliliter of each dilution then was inoculated into the allantoic chamber of each of five, 10- or 11-day embryonating eggs. The eggs were incubated at 37 C. and candled twice daily for seven days. Fluids from all embryos of which the specificity of the death was questioned were tested for bacterial contamination and for hemagglutinative activity. Hemagglutination tests using chicken erythrocytes were made by the method of Salk.[10] The 50 per cent embryo lethal dose (e.l.d.$_{50}$) was calculated by the method of Reed and Muench.[9]

Virus Cultures.—The virus was grown in chorioallantoic membranes (CAM) supended in Tyrode's solution. A CAM was removed from a 10- or 11-day embryonating egg, washed three times in bottles containing 200 ml. of saline solution, and then suspended in 20 ml. of Tyrode's solution.

TABLE 1—Stability of Newcastle Disease Virus in Tyrode's Solution, Containing 40 μ per Milliliter of Various Antimetabolites, at 37 C. After 26 Hours

Antimetabolite (40 μ/ml.)	e.l.d.$_{50}$/ml. (at 37 C. after 26 hr.)
Control 1 (no inhibitor)	$10^{2.9}$
Control 2 (no inhibitor)	$10^{2.8}$
Proflavin	$10^{2.5}$
2,4-dinitrophenol	$10^{2.5}$
Sodium azide	$10^{2.4}$
Urethane	$10^{2.6}$

Initial virus titer was $10^{6.3}$ e.l.d.$_{50}$ per milliliter.

One milliliter of virus containing approximately $10^{2.3}$ e.l.d.$_{50}$ was used to infect each membrane. A small infective dose was used so that the virus remaining in the supernatant fluid would be negligible. The cultures then were incubated at 37 C., and samples of the culture fluid were removed at appropriate intervals and stored at −20 C. until virus content could be titrated. Only the number of infective units of virus in the extracellular fluid was determined.

Preparation and Sterilization of Metabolic Inhibitors. Solutions of the inhibitors [2,4-dinitrophenol (DNP), urethane, proflavin, sodium azide, sodium malonate, and sodium acetate] were made in distilled water or saline solution to give an initial concentration of 1,000 μ per milliliter or a 1 M solution. Sterilization of each compound was accomplished by filtration through a Seitz pad or by heating the solution at 80 C. for 25 minutes, depending upon the stability of the compound. The malonic acid was neutralized with 1 N NaOH. Further dilutions of the sterilized solutions were made in Tyrode's solution to obtain the desired concentrations.

Toxicity of the Antimetabolites.—Each antimetabolite was pretested at several levels for toxicity to the cells of the CAM which have the capacity to produce virus. Only those concentrations were used which did not appear to damage the cells of the CAM and which were not virucidal.

Effect of Antimetabolites on Growth of NDV in CAM.—A washed CAM was placed in 23 ml. of

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Based in part on a thesis submitted by the senior author to the graduate school of the University of Wisconsin in partial fulfillment of the requirements for the Ph.D. degree.

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* Obtained from the Newcastle disease virus repository at the University of Wisconsin, Madison.

Tyrode's solution and infected with 1 ml. of NDV, which contained approximately $10^{2.3}$ e.l.d.$_{50}$. One milliliter of appropriate dilutions of each antimetabolite, expressed as a final concentration as shown in the tables, was added to the culture. All concentrations of a given metabolite were usually tested in triplicate.

Glucose Determinations.—Glucose concentrations were determined by the anthrone method of Morris.[7]

RESULTS

The stability of NDV in the presence of 40 μ per milliliter of proflavin, DNP, sodium azide, and urethane is shown (table 1). Its stability in the presence of these inhibitors is less than in the control solution, but the differences are small and probably not significant. Also, 0.01 M solutions of sodium malonate, sodium fluoroacetate, and urethane were not virucidal (table 2), since

TABLE 2—Stability of Newcastle Disease Virus in Tyrode's Solution Containing 0.01 M Concentrations of Different Antimetabolites

0.01 M solutions	e.l.d.$_{50}$/ml. (at 37 C. after 26 hr.)
Control (no antimetabolite)	$10^{5.2}$
Sodium fluoroacetate	$10^{5.2}$
Sodium malonate	$10^{4.8}$
Urethane	$10^{4.8}$

Virus suspensions were held at 37 C. for 26 hours. Initial virus titer was $10^{7.4}$ e.l.d.$_{50}$ per milliliter.

the stability of NDV in Tyrode's solution after incubation at 37 C. for 26 hours was approximately the same as it was in Tyrode's solutions which contained the 0.01 M concentrations of sodium malonate, sodium fluoroacetate, and urethane.

Knowing that the compounds were not virucidal, it was necessary next to test their toxicity for cells of CAM. This was done by incubating the CAM in the presence of the various compounds for 24 or 36 hours. After washing to remove any of the compounds adhering to the membrane, the membrane was infected with virus. If the quantity of virus produced by these treated membranes was significantly lower than that produced by the untreated CAM, it could be concluded that the antimetabolite had destroyed cells which were capable of producing virus. If, however, the titer of the virus produced by treated cells was not reduced, the cells of the membrane that are host to the virus must not have been injured. Urethane, proflavin, or sodium azide at a concentration of 40 μ per milliliter did not damage the cells of the

TABLE 3—The Growth of Newcastle Disease Virus in Chorioallantoic Membranes Which Had Been Preincubated in Tyrode's Solution Containing Different Antimetabolites

Antimetabolites and concentrations	e.l.d.$_{50}$/ml. (at 37 C. after 24 hr.)
Control (no inhibitor)	$10^{5.8}$
2,4-dinitrophenol (40 μ/ml.)	$10^{2.4}$
Proflavin (40 μ/ml.)	$10^{2.4}$
Urethane (40 μ/ml.)	$10^{4.2}$
Sodium azide (40 μ/ml.)	$10^{2.2}$

Preincubation was at 37 C. for 26 hours. Each membrane was washed and infected with $10^{1.3}$ e.l.d.$_{50}$ of Newcastle disease virus and reincubated at 37 C. for an additional 24 hours.

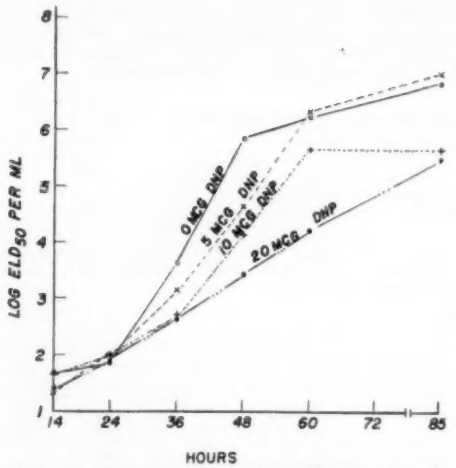
CAM which support the growth of NDV. Approximately the same virus titer was produced by the CAM, whether it had or had not been incubated in Tyrode's solution containing 40 μ per milliliter of the antimetabolites (table 3). A 0.01 M solution of each of the antimetabolites (urethane, sodium fluoroacetate, or sodium malonate) did not produce more damage to CAM cells than did the Tyrode's solution alone (table 4).

When the metabolic inhibitors were added to Tyrode's solution in which virus-infected chorioallantoic membranes were placed, the yield of virus was affected. The growth of NDV decreased as the concentration of DNP was increased (graph 1). The decrease was most evident in the later periods of growth, *i.e.*, after about the forty-ninth hour. The apparent failure to demonstrate inhibition of virus growth during the early periods of incubation may be ascribed to insensitivity in the method of assay or to a delay in the relay of effect through the host cell, the latent phase of virus growth, or transitory adaptation of the host cell to the inhibitor. The maximum degree of inhibition was exerted between the thirty-sixth and the sixtieth hour of growth, while at the eightieth hour,

TABLE 4—The Growth of Newcastle Disease Virus in Chorioallantoic Membranes Which Had Been Preincubated in Tyrode's Solution Containing Different Antimetabolites

Antimetabolites and concentrations	e.l.d.$_{50}$/ml. (at 37 C. after 48 hr.)
0.01 M sodium malonate	$10^{7.2}$
0.01 M urethane	$10^{7.3}$
0.01 M fluoroacetate	$10^{7.0}$
Controls (no antimetabolites)	$10^{7.2}$

Preincubation was at 37 C. for 26 hours. Each membrane then was washed and infected with $10^{2.0}$ e.l.d.$_{50}$ of Newcastle disease virus and reincubated in fresh Tyrode's solution at 37 C. for 48 hours.



Graph 1—Growth of Newcastle disease virus in chorioallantoic membranes isolated in Tyrode's solution that contained different concentrations of 2,4-dinitrophenol (DNP).

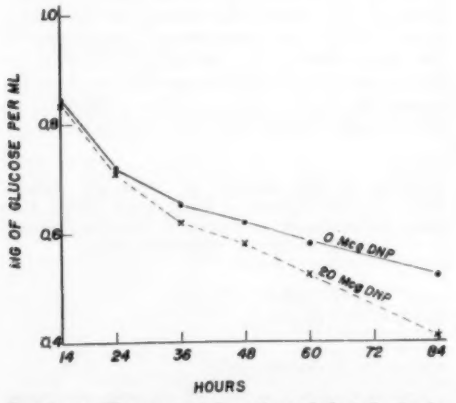
growth of the NDV in the two highest concentrations of DNP approached a maximum stationary phase. These titers were much lower than the titer of the control culture.

Complete inhibition occurred in cultures containing 40 μ per milliliter of DNP. Viral multiplication could not be detected at any stage of incubation. It is possible that the respiration of the cells was suppressed. However, a decrease in the glucose concentration at this level of DNP indicated that the respiration of the cells persisted.

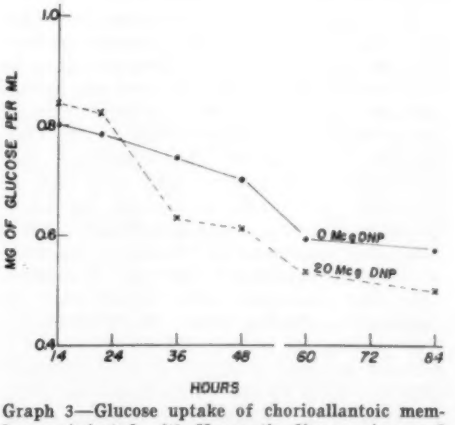
Concentrations of DNP from 5 to 40 μ per milliliter retarded the growth of NDV in chorioallantoic tissue culture. This retardation may have been due to the temporary interference of transfer of energy through high-energy phosphate bonds within the membrane cells. These results with NDV are in agreement with those of Eaton and Perry,[4] who also found that DNP at concentrations of 5 to 30 μ per milliliter inhibited the growth of influenza virus in chorioallantoic tissue culture. However, they found that the inhibition of the virus was reversible within 48 hours, while, in our experience, the inhibition of growth reversed only after a much longer period. The difference might be explained by the fact that Eaton and Perry grew the influenza virus in shake flask tissue cultures, while the NDV-infected tissue cultures were incubated in a quiescent state with less accumulation of cell debris and lower rate of respiration.

Study of the glucose utilization revealed that DNP stimulated glycolysis of both infected and noninfected tissues suspended in Tyrode's solution (graphs 2, 3). Eaton[3] and Ackermann and Johnson[2] felt that stimulation of glycolysis of CAM by DNP was an expression of low phosphate esterification. Our results are in agreement.

Urethane did not inhibit the growth of virus in CAM. Since the concentration of 40 μ per milliliter of urethane may not have been great enough to inhibit virus growth, an experiment was performed with



Graph 2—Glucose uptake of noninfected chorioallantoic membranes suspended in Tyrode's solution with and without 2,4-dinitrophenol (DNP).



Graph 3—Glucose uptake of chorioallantoic membranes infected with Newcastle disease virus and suspended in Tyrode's solution with and without 2,4-dinitrophenol (DNP).

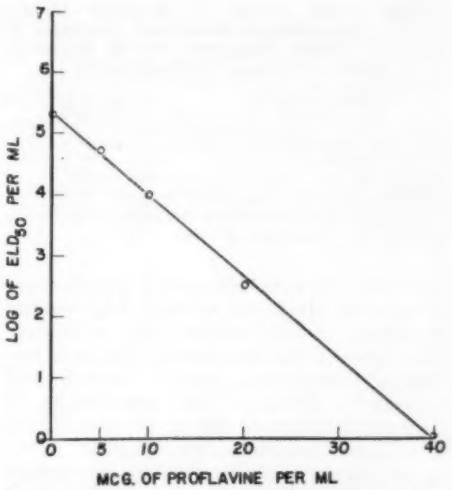
a greater concentration (0.01 M solution). Inhibition of the growth of NDV was not produced.

Both sodium azide and proflavin inhibited virus growth to about the same extent (graphs 4, 5). Forty micrograms per milliliter of each of these inhibitors produced about a 4 log decrease in the e.l.d.$_{50}$ of NDV in the CAM culture after 48 hours. The results with sodium azide confirm the conclusions reached in the DNP studies, since sodium azide also uncouples high-energy bonds. Sodium azide probably interferes with the energy produced by the host cell which is channeled to the production of virus. According to Eaton,[3] the reaction of proflavin is similar to that produced by DNP on virus growth.

Tyrode's solutions containing 0.01 M concentrations of urethane or sodium fluoroacetate or sodium malonate failed to inhibit the production of NDV in CAM suspended in these solutions (table 5). It could be postulated that the energy for synthesis of NDV does not come from the functioning of the Krebs cycle, since neither malonate nor fluoroacetate interfered with virus production in CAM. However, it must be remembered that only a 0.01 M solution of these antimetabolites was used, *i.e.*, the 0.01 M solution might not be concentrated enough to inhibit the functioning of the Krebs cycle in the CAM and, consequently, would not interfere with virus synthesis.

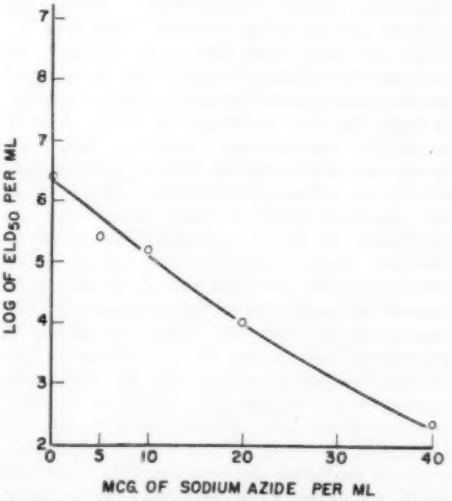
DISCUSSION

During the eclipse phase of viral growth, the metabolism of the host is readied for the production of viral material. The redirected metabolism is of two types: (1) catabolic, or that concerned with providing energy for virus synthesis, and (2) anabolic, or that concerned with the synthesis of proteins and nucleic acids found in the virus particles. We have attempted to interrupt the flow of energy to the synthetic systems of the host cell at several points in an effort to inhibit viral growth. Morgan[6] has pointed out that inhibition of viral growth by interference with specific metabolic systems must be restricted to those that are not also necessary for the immediate integrity of the host cell. This restriction was observed, as the concentrations of the antimetabolites used were not lethal for NDV and did not produce a deleterious effect measurable by these criteria on the cells of the CAM that support the growth of the virus.



Graph 4—Production of Newcastle disease virus in chorioallantoic membranes isolated in Tyrode's solution which contained different concentrations of proflavin and was incubated at 37 C. for 48 hours.

Concentrations of DNP from 5 to 40 μ per milliliter retarded the growth of NDV in chorioallantoic tissue. The retardation may



Graph 5—Production of Newcastle disease virus in chorioallantoic membranes isolated in Tyrode's solution containing various concentrations of sodium azide and incubated at 37 C. for 48 hours.

TABLE 5—The Growth of Newcastle Disease Virus in Chorioallantoic Membranes Suspended in Tyrode's Solution Containing 0.01 M Concentrations of Different Antimetabolites

Antimetabolites (0.01 M)	e.l.d.$_{50}$/ml. (at 37 C. after 36 hr.)
Controls (no inhibitor)	$10^{6.2}$
Sodium malonate	$10^{5.8}$
Fluoroacetate	$10^{5.8}$
Urethane	$10^{5.8}$

Incubation was at 37 C. for 36 hours. Initial virus titer was $10^{1.3}$ e.l.d.$_{50}$ per milliliter.

have been due to temporary interference of transfer of energy through high-energy phosphate bonds within the membrane cells, since it has been shown that DNP uncouples high-energy bonds (Loomis and Lipman [5]). However, the retardation of viral growth may be due to the stimulation of a latent adenosintriphosphatase (ATP) by DNP, found in chorioallantoic membranes by Ackermann.[1]

Increased glycolysis in CAM suspended in Tyrode's solution containing DNP may be a sign of low phosphate esterification produced by DNP, since Ackermann and Johnson [2] have shown that this stimulation in CAM activity is accompanied by a decreased rate of phosphorylation and an accelerated liberation of inorganic phosphate from intact host cells.

Apparently, glucose is not required for growth of NDV under "aerobic" conditions whereas, under "anaerobic" conditions, glucose seems to be required (Zuschek [11]). This indicates that the CAM contains an endogenous substrate which can be utilized under aerobic conditions to produce enough energy for the synthesis of virus. Under anaerobic conditions, however, the CAM needs an additional energy-supplying substrate for viral reproduction. Moulder *et al.*[8] obtained evidence that energy for the synthesis of feline pneumonitis virus in isolated chicken embryo yolk sac is probably supplied by high-energy bonds generated by oxidation of the endogenous substrates in the yolk sac. The retardation of growth of NDV in CAM by DNP might be due to the dissipation of energy of preformed high-energy compounds, such as creatin phosphate or ATP. This seemed to be the situation, because Krebs' cycle inhibitors, such as malonate and fluoracetate, did not have to show a deleterious effect upon growth of NDV. If a preformed high-energy compound would supply energy, the Krebs cycle might not be required, at least in the initial stages of viral multiplication. This hypothesis fits with the observation, since antimetabolites, such as DNP and sodium azide, which interfere with the utilization of high-energy compounds also interfere with the growth of NDV, while other antimetabolites, e.g., malonate and fluoroacetate, which inhibit production of energy through the Krebs cycle, have no apparent influence upon the growth of NDV in CAM tissue cultures. Eaton [3] suggested that the action of proflavin and DNP in inhibiting viral reproduction are similar. This gives additional support to the proposed catabolic mechanisms for synthesis of virus, since proflavin, like DNP, was a potent inhibitor of the growth of NDV in tissue culture.

SUMMARY

The effects of various concentrations of antimetabolites on the growth of Newcastle disease virus (NDV) in chorioallantoic membranes (CAM) suspended in Tyrode's solution were studied.

The highest concentrations of the various antimetabolites used [2,4-dinitrophenol (DNP), urethane, sodium azide, proflavin, sodium malonate, and sodium fluoroacetate] were not lethal for NDV and apparently did not damage the cells of the CAM which support virus growth.

At concentrations of 5 to 40 μ per milliliter, DNP retarded the growth of NDV in CAM cultures. The growth of NDV decreased as the concentration of DNP in the Tyrode's solution was increased. Glycolysis was stimulated in both infected and noninfected tissue by the presence of DNP.

Proflavin and sodium azide, at concentrations of 5 to 40 μ per milliliter, were effective in inhibiting the production of NDV.

No significant inhibition of the growth of NDV was demonstrable in Tyrode's solutions that contained 0.01 M of urethane, sodium malonate, or sodium fluoroacetate.

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The Complement-Fixation Test in the Diagnosis of Sarcosporidiosis

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IN ANY generalized infection such as sarcosporidiosis, the clinical signs tend to be obscure and nonspecific in character. Diagnosis is, therefore, difficult and frequently impossible in the living animal.

In 1944, Kean and Grocott [6] concluded that, in the absence of inoculation and serological studies, the absolute identification of Sarcocystis and Toxoplasma is impossible.

Muhlpfordt,[7] in 1951, showed that the Sabin-Feldman dye test gives positive results with both Toxoplasma and Sarcocystis. Awad and Lainson,[4] in 1954, confirmed Muhlpfordt's findings, but added that the complement-fixation test can differentiate between these infections.

Awad [1] described a new dye test for the diagnosis of sarcosporidiosis, but the test was nonspecific. He showed [2] the lack of specificity of the Sabin-Feldman dye test. He also described [3] an intradermal test for the diagnosis of sarcosporidiosis, but concluded that, while the test has little diagnostic value, it can be used as a screening test.

This paper presents results with which to evaluate the complement-fixation (CF) test in the diagnosis of sarcosporidiosis.

MATERIALS AND METHODS

The antigen used was prepared as described by Awad and Lainson [4] and was similar to that used by Awad [5] for the intradermal test.

The complement and hemolytic serum were obtained from one source* and the preserved ovine cells from another.†

The anticomplementary effect of the antigen is shown (table 1). The tubes were incubated in a water bath for 30 minutes at 37 C. Hemolysis occurred in all tubes except tube 7, which was the corpuscle control, indicating that the antigen prepared in this way is not anticomplementary.

The hemolytic activity of the antigen is shown (table 2). No hemolysis occurred in any of the tubes, indicating that the antigen prepared in this way is nonhemolytic.

The antigen was titrated against the serum of an artificially immunized guinea pig (table 3). This was a typical chessboard experiment, and the results can be most conveniently represented graphically (graph 1). There are no optimum points in the 50 per cent hemolysis curve because there is no zone phenomenon. Point B was chosen as the antigen titer because it is the nearest to the maximum serum titer and it permits the use of small amounts of antigen for the purpose of economy.

Technique.—The technique adopted was similar to that used for the Wassermann reaction. A constant volume (uni. volume per reagent is 0.11 ml.) method was used.

All serums were incubated previously at 56 C. for 30 minutes. Complement was used at 3 m.h.d. (minimum hemolytic dose) per unit volume, and the antigen at a 1:40 dilution. The constituents were added in the following order: 0.11 ml. of inactivated serum, 0.11 ml. of complement containing 3 m.h.d., and 0.11 ml. of antigen at a dilution of 1:40. The mixture was shaken and placed in a 37-C. water bath for one hour. Then 0.11 ml. of the hemolytic system was added and the tubes were shaken well and again placed in the 37-C. water bath. Provisional readings were made when the serum control tubes had all hemolyzed, and final readings were taken when the normal serum control, put up with every set of tests, showed complete hemolysis. Positive control serum also was put with each set of tests.

Microscopic Examination of Ovine Tissues for Sarcocystis.—The sites selected for microscopic

TABLE 1—Anticomplementary Effect of the Antigen

Tube (No.)	Antigen (0.11 ml.)	Complement (3 m.h.d.)	Saline solution	Fixation	Sensitized erythrocytes
1	1:5	0.11 ml.	0.11 ml.	In	0.11 ml.
2	1:10	0.11 ml.	0.11 ml.	water	0.11 ml.
3	1:20	0.11 ml.	0.11 ml.	bath	0.11 ml.
4	1:40	0.11 ml.	0.11 ml.	for	0.11 ml.
5	1:80	0.11 ml.	0.11 ml.	1	0.11 ml.
6	1:160	0.11 ml.	0.11 ml.	hour at	0.11 ml.
7	.....	.....	0.33 ml.	37 C.	0.11 ml.

From the Veterinary Research Division, Ministry of Animal Resources, Khartoum, Sudan.

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† Medical Research Institute, London, England.

examinations were the esophagus, heart, and diaphragm muscles. The esophagus was the primary site for macroscopic cysts, while scrapings from the muscle of the heart, diaphragm, and esophagus were placed separately on slides in normal saline solution, under cover slips, and examined under the 2/3 objective. In view of the obvious limitations of such methods, the description, "animals in which no cysts were found," was used in preference to "negative" when cysts could not be found by these means.

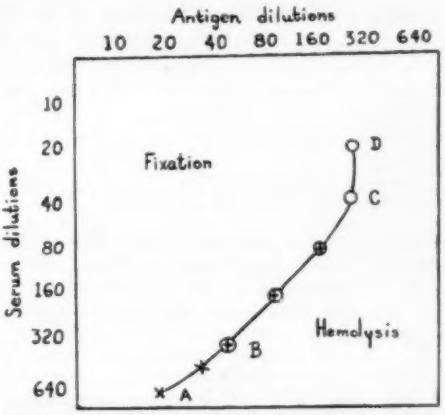
OBSERVATIONS

Specificity of the CF Test.—The test was carried out on 45 ovine and two human serum samples. In addition to Sarcocystis antigen, Toxoplasma antigen prepared in a similar way was used. The results of the test, as well as the incidence of the presence of Sarcocystis, are shown (table 4). Of 25 sheep with detectable infections, 20 had positive reactions to the CF test when Sarcocystis antigen was used, but negative reactions when Toxoplasma antigen was used. Of 20 sheep in which no cysts could be found, 13 had positive reactions to the CF test when Sarcocystis antigen was used, and 1 had a positive reaction when Toxoplasma antigen was used. The two human beings with detectable infections had positive reactions only when Sarcocystis antigen was used.

TABLE 2—Hemolytic Activity of the Antigen

Tube (No.)	Antigen (0.11 ml.)	Sensitized erythrocytes	Saline solution (ml.)	Incubation
1	undiluted	0.11 ml.	0.22	30 minutes
2	1:5	0.11 ml.	0.22	at 37 C.,
3	1:10	0.11 ml.	0.22	water bath

The CF Test on Ovine Serums for the Detection of Natural Infections.—Serums were collected from 245 sheep of different ages and sexes and from different locations. Of 134 animals in which sarcosporidiosis was detected, either macroscopically or microscopically, 113 had positive reactions to the CF test. Of 111 animals in which cysts could not be found, 58 had positive reactions. The highest titer obtained was 1:80 and, from a correlation between the presence of the infection and the results of the CF test, it was possible to suggest that a titer of 1:10 is significant.



Graph 1—Results of antigen titration with short fixation. Complement was used at 3 m.h.d. per unit volume. The curve represents all mixtures giving 50 per cent hemolysis. These points were obtained from data in table 3 (circles from horizontal rows, crosses from vertical rows) and are joined by line ABCD. All mixtures to the left (or inside) of the 50 per cent hemolysis curve showed complete fixation, while all to the right (or outside) gave complete hemolysis. The line AB represents the maximum serum titer, and point B is taken as the optimum antigen titer (1:40).

Serum Titer.—The rise and fall in the serum titer of an experimentally infected lamb was investigated. The highest titer (1:80) was obtained 184 days postinfection, after which it started to fall but was still detectable up to 300 days postinfection.

The CF Test on Human Serums.—During this investigation, the CF test was carried out on serums from two adult men

TABLE 3—Results of Antigen Titration

Serum dilutions	Antigen dilutions						
	1:10	1:20	1:40	1:80	1:160	1:320	1:640
1:10	0	0	0	0	0	0	0
1:20	0	0	0	0	0	+++	++++
1:40	0	0	0	0	0	+++	++++
1:80	0	0	0	0	++	++++	++++
1:160	0	0	0	++	+++	++++	++++
1:320	0	+	++	+++	+++	++++	++++
1:640	+++	+++	++++	++++	++++	++++	++++

Antigen control (1:20) = ++++; serum control (1:20) = ++++; and corpuscle control = 0.
++++, +++, ++, and + = 100, 75, 50, and 25 per cent hemolysis, respectively; 0 = no hemolysis.

TABLE 4—Results of the Specificity of the CF Test

No. tested	Presence of cysts	No. positive to CF test using Sarcocyst antigen	No. positive to CF test using Toxoplasma antigen
45 sheep	25 positive	20	0
	20 negative	13	1
2 human beings	2 positive	2	0

known to be infected with Sarcocystis (Pugh [8] and Goodbody [5]). Both serum samples were positive to the test; the titer of the first was 1:80, and of the second, 1:40.

DISCUSSION

The lack of a reliable method for diagnosing sarcosporidiosis prompted this investigation for the development of complement-fixing antibodies.

The first problem was to determine whether a Sarcocystis-infected tissue could be found which would yield an extract that would fix complement in the presence of a known immune serum. The antigen finally found to give specific complement-fixing antibodies was then demonstrated by testing the serums of naturally infected animals.

A frozen and thawed extract of Sarcocystis yielded the most potent complement-fixing antigen. This antigen proved to be highly specific and, since it was wholly derived from the parasite, had no anticomplementary effect. This antigen detected the infection in other animals, including man. The frozen antigen, kept at —70 C., was maintained for over a year without deterioration.

It is believed that this investigation is the first attempt to use the CF test in the diagnosis of sarcosporidiosis; it has been shown to be a reliable and a practical serological method for that purpose. Of 245 sheep tested, 172 were positive to the CF test; 58 in the absence of detectable infections. There were no reactions in 21 animals with detectable cysts. As the infection is not restricted to the organs examined, it is inferred that 193 of the 245 sheep were infected with Sarcocystis. If so, the average rate of failure of microscopic examination is 58/193 (30.5%) and the average rate of failure of the CF test is 21/193 (10.9%); *i.e.*, the failure of microscopic examination to detect the infection is about three times that of the CF test.

Of the 21 animals with detectable cysts which failed to react to the CF test, 14 were old ewes with macroscopic cysts, some of which had ceased to be active, and the titer of the complement-fixing antibodies might have fallen to a level at which it was no longer detectable. The CF test may be most useful in the rapid diagnosis of active infection, although it may sometimes fail.

This investigation has indicated that the CF test also may be reliable in the diagnosis of sarcosporidiosis in human beings and, thus, might be used to work out the true incidence of this infection in human beings.

There had been no reliable diagnostic method previously, and no serious attempt at differential diagnosis, especially from closely related infections such as toxoplasmosis. Awad and Lainson [4] concluded that, as far as the CF test is concerned, no demonstrable relationship exists between Sarcocystis and Toxoplasma infections.

SUMMARY

1) This study describes the use of the complement fixation (CF) test as a method for diagnosing sarcosporidiosis.

2) A frozen and thawed extract of the cysts yielded the most potent complement-fixing antigen.

3) The test is specific in the diagnosis of sarcosporidiosis, and a titer of 1:10 is significant.

4) While the test may sometimes fail, it is most useful in the diagnosis of active infections.

5) It is concluded that the CF test is a reliable, practical method for diagnosing sarcosporidiosis, and also could be used to differentiate this infection from toxoplasmosis.

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The Effects of Age and Temperature on the Survival of Oocysts of *Eimeria Stiedae*

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SEVERAL FACTORS, including oxygen tension, bacterial action, desiccation, and extremes in temperature, are responsible for the survival or destruction of coccidial oocysts and govern the rate of sporulation. The absolute temperature ranges in which the oocysts survive and the upper and lower limits of these ranges have not been determined.

Edgington and Broerman[2] found that the oocysts of avian coccidiosis were viable for two years when stored in a 2.5 per cent solution of potassium dichromate at 3.3 to 5.5 C. Goodrich[3] reported that *Eimeria tenella* oocysts survived for 24 to 25 months at room temperature in a solution of 2.5 per cent potassium dichromate. Patterson[4] reported that the oocysts of *E. tenella*, mixed with soil, would survive for at least 12 weeks at a constant temperature of −1.0 C.

This experiment was an attempt to determine (1) the effect of prolonged storage on the viability of the oocysts of *Eimeria stiedae* and (2) the temperature at which viability ceased or sporulation failed to take place.

MATERIALS AND METHODS

Five samples of unsporulated *E. stiedae* oocysts were used in the first tests. Samples 1, 4, and 5 were from liver lesions and samples 2 and 3 were from gallbladders of experimentally infected rabbits. The oocysts had been stored in normal saline solution in screw-capped bottles at 5 C. for 3.5 to 4.5 years. The oocysts then were sporulated and each 56-day-old test rabbit, weighing approximately 3.75 lb., was given about 25,000 oocysts through a rubber catheter passed into the stomach. The animals were killed and examined for infections 22 days later.

Oocysts also were collected from the gallbladders of several experimentally infected rabbits. After they were washed in saline solution, half the oocysts were sporulated in a 2 per cent solution of potassium dichromate. Portions of the unsporulated and sporulated oocysts were suspended in 5 per cent glycerol, in 1 per cent gelatin, and in 1 per cent milk solutions. Suspensions of oocysts then were placed in shell vials, frozen rapidly, and kept at a temperature of −25 C. for one, seven, 14, and 30 days. At each interval, vials of both sporulated and unsporulated oocysts from the three diluents were removed from the freezer and allowed to thaw. The unsporulated oocysts were placed in a 2 per cent solution of potassium dichromate for sporulation. Aproximately 5,000 sporulated oocysts were given to each of several 56-day-old rabbits which were examined 26 days later for evidence of infection.

RESULTS

When removed from storage and placed in a 2 per cent solution of potassium dichromate, the *E. stiedae* oocysts sporulated normally and within the time limit indicated by Becker.[1] The oocysts survived for 4.5 years at 5 C. (table 1) and produced typical liver lesions in the test rabbits. Oocysts also were recovered from the bile tracts and from the contents of the ceca. The test animal given sample 1 died after

TABLE 1—Viability of Unsporulated Eimeria Stiedae Oocysts Stored at 5 C., then Sporulated and Fed to 56-Day-Old Rabbits

Sample No.	Time in storage	Dose of oocysts	Results of animal inoculations		
			Liver lesions	Oocysts recovered from	
				Gallbladder	Cecum
1	4 yr., 6 mo.	25,000	Neg.	Neg.	Pos.
2	4 yr., 6 mo.	25,000	Pos.	Pos.	Pos.
3	4 yr., 4 mo.	25,000	Pos.	Pos.	Pos.
4	4 yr., 0 mo.	25,000	Pos.	Pos.	Pos.
5	3 yr., 6 mo.	25,000	Pos.	Pos.	Pos.

The rabbit given sample 1 died after ten days; the other test rabbits were killed and examined on the twenty-second day after the oocysts were given.

From the U.S. Rabbit Experiment Station, Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Fontana, Calif.

TABLE 2—Viability of Sporulated Eimeria Stiedae Oocysts Stored at —25 C., then Fed to 56-Day-Old Rabbits

Diluent	No. of days in storage	Animal No.	Dose of oocysts	Results of animal inoculations		
				Liver lesions	Oocysts recovered from	
					Gallbladder	Cecum
5 per cent glycerol	1	1	5,000	Neg.	Neg.	Pos.
	7	2	5,000	Pos.	Pos.	Pos.
	14	3	5,000	Pos.	Pos.	Pos.
	30	4	5,000	.....	.....	.....
1 per cent gelatin	1	5	5,000	Pos.	Pos.	Pos.
	7	6	5,000	Pos.	Pos.	Pos.
	14	7	5,000	Neg.	Neg.	Neg.
	30	8	5,000	Neg.	Neg.	Neg.
1 per cent milk	1	9	5,000	Pos.	Pos.	Pos.
	7	10	5,000	Pos.	Pos.	Pos.
	14	11	5,000	Neg.	Neg.	Pos.
	30	12	5,000	Pos.	Pos.	Pos.

Rabbit 4 died during the administration of the oocysts. Rabbit 1 died after 17 days; 7 and 8 after ten days; 11 after 14 days; the remainder were killed and examined on the twenty-sixth day after the oocysts were given.

ten days and no lesions were found; oocysts were found in the cecum but not in the gallbladder.

When the vials containing the sporulated and unsporulated oocysts were allowed to thaw, the oocysts appeared normal, morphologically, and the cell walls and structures were intact. However, the unsporulated oocysts placed in a 2 per cent solution of potassium dichromate failed to sporulate during a 14-day observation period.

Sporulated oocysts kept at −25 C. for one to 30 days were viable and produced typical liver lesions when given to test animals (table 2). The benefit of one diluent over the other was not indicated. Test rabbits 1, 7, 8, and 11 died before liver lesions had developed and, when death occurred after ten days, oocysts were found in the cecal contents. If the dosage of oocysts had been larger, liver lesions might have been found in animal 1 at 17 days.

SUMMARY

1) Unsporulated *Eimeria stiedae* oocysts, which had been stored at 5 C. in saline solution for 3.5 to 4.5 years, sporulated normally when placed in a 2 per cent solution of potassium dichromate and produced typical liver lesions in experimental animals.

2) Unsporulated oocysts which had been held at −25 C. in 5 per cent glycerol, in 1 per cent gelatin, and in 1 per cent milk solutions for one to 30 days failed to sporulate within a 14-day period.

3) Sporulated oocysts which had been held at −25 C. in 5 per cent glycerol, in 1 per cent gelatin, and in 1 per cent milk solutions for one to 30 days were viable and produced typical liver lesions in test animals.

References

[1] Becker, E. R.: Coccidia and Coccidiosis of Domesticated Game and Laboratory Animals and of Man. Collegiate Press, Inc., Ames, Iowa (1934): 77–88.

[2] Edgington, B. H., and Broerman, A.: Longevity of Avian Coccidial Oocysts. Ann. Rep. Ohio Agric. Exper. Sta., Bull. 470, (1929–30): 186–187.

[3] Goodrich, H. P.: Coccidian Oocysts. Parsitology, *36*, (1944): 72–79.

[4] Patterson, F. D.: Studies on the Viability of *Eimeria Tenella* in Soil. Cornell Vet., *23*, (1933): 232–249.

THE INDEX

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